Novel Technologies for detection, production and screening of stress tolerant bifidobacteria

Author(s):
Reimann, Sebastian

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NOVEL TECHNOLOGIES FOR DETECTION, PRODUCTION AND SCREENING OF STRESS TOLERANT BIFIDOBACTERIA

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presented by

SEBASTIAN REIMANN
Dipl. Natw. ETH

Born April 24, 1979
Citizen of Oberhof AG

Accepted on the recommendation of

Prof. Dr. Christophe Lacroix, examiner
Prof. Dr. Leo Meile, co-examiner
Prof. Dr. Paul Ross, co-examiner

2009
Table of contents

TABLE OF CONTENTS .................................................................................................................. I

ABBREVIATIONS .......................................................................................................................... V

SUMMARY ....................................................................................................................................... VI

ZUSAMMENFASSUNG ..................................................................................................................... IX

1. INTRODUCTION .................................................................................................................... 1

1.1 Human gastrointestinal tract microbiota .............................................................................. 1

1.2 Bifidobacterium ..................................................................................................................... 3
  1.2.1 General characteristics ................................................................................................. 3
  1.2.2 Metabolism of Bifidobacteria ....................................................................................... 4
  1.2.3 Role of Bifidobacteria in the GIT ................................................................................ 5
  1.2.4 Transcriptomics of Bifidobacterium longum NCC2705 .............................................. 6

1.3 Probiotic concept ................................................................................................................... 7

1.4 Health promoting effects related to probiotics ................................................................. 9

1.5 Potential risks associated with probiotics .......................................................................... 2

1.6 Technological requirements of probiotic bacteria .......................................................... 13

1.7 Technologies to improve cell viability and yields ............................................................ 15
  1.7.1 Production technologies .............................................................................................. 15
    1.7.1.1 Optimization of growth medium ............................................................................. 15
    1.7.1.2 Stress adaptation .................................................................................................... 16
    1.7.1.3 Immobilized cell (IC) technology in continuous cultures ..................................... 20
      1.7.1.3.1 Principle of cell immobilization ........................................................................ 20
      1.7.1.3.2 Techniques of cell immobilization ................................................................. 21
      1.7.1.3.3 Physiological changes of immobilized cells .................................................. 22
      1.7.1.3.4 Advantages of continuous IC cultures ........................................................... 23
    1.7.2 Stabilization of probiotics ............................................................................................ 26
      1.7.2.1 Microencapsulation ............................................................................................. 26
      1.7.2.2 Spray drying ....................................................................................................... 27
      1.7.2.2.1 Principle of spray drying ................................................................................. 28
      1.7.2.2.2 Parameters affecting cell viability during spray drying ................................... 31
      1.7.2.2.3 Parameters affecting storage of dried powders ............................................. 36
      1.7.2.2.4 Rehydration .................................................................................................... 38
    1.7.3 Enumeration of viable probiotic cells .......................................................................... 39
Table of contents

3.4 Results .................................................................................................................................................. 81
  3.4.1 Morphology of *B. longum* NCC2705 ............................................................................................ 81
  3.4.2 Calibration curves .......................................................................................................................... 82
  3.4.3 Heat test ......................................................................................................................................... 84
  3.4.4 Rifampicin test ............................................................................................................................... 85
  3.4.5 Viable cells quantification of cell aggregates .................................................................................. 86

3.5 Discussion ............................................................................................................................................ 87

4 DEVELOPMENT OF A RAPID SCREENING PROTOCOL FOR SELECTION OF
*BIFIDOBACTERIUM LONGUM* STRAINS RESISTANT TO SPRAY DRYING AND
POWDER STORAGE ................................................................................................................................. 90

4.1 Abstract ................................................................................................................................................ 91

4.2 Introduction ......................................................................................................................................... 92

4.3 Material and Methods ....................................................................................................................... 94
  4.3.1 Bacterial strains and culture conditions ....................................................................................... 94
  4.3.2 Preparation of bacterial suspensions used for survival tests ...................................................... 95
  4.3.3 Preparation of strain mix ............................................................................................................. 95
  4.3.4 Spray drying ................................................................................................................................... 96
  4.3.5 Storage test .................................................................................................................................... 96
  4.3.6 Determination of water activity and moisture content ............................................................... 97
  4.3.7 Viable cell counts in spray dried powders .................................................................................. 97
  4.3.8 Identification of best surviving strains using RAPD ................................................................. 98

4.4 Results ................................................................................................................................................ 99
  4.4.1 Strain survival and selection during the first selection batches ............................................... 99
  4.4.2 Strain survival and selection during the second selection round ............................................. 104
  4.4.3 Validation of selection procedures using single strain preparations ..................................... 106

4.5 Discussion .......................................................................................................................................... 107

5 GENERAL CONCLUSIONS AND OUTLOOK ...................................................................................... 111

5.1 General conclusions .......................................................................................................................... 111

5.2 Outlook and perspectives .................................................................................................................. 114

6 APPENDIX ........................................................................................................................................... 117

6.1 Supplementary material from Chapter 4 ........................................................................................ 117

6.2 Genome expression analysis of *B. longum* NCC2705 produced during continuous culture with
immobilized cells and batch culture ....................................................................................................... 118
  6.2.1 Background .................................................................................................................................. 118
  6.2.2 Material and methods .................................................................................................................. 118
  6.2.3 Results and discussion ................................................................................................................. 121
    6.2.3.1 Comparison of cells from ICC with batch cultures .............................................................. 121
    6.2.3.2 Comparison between ICC1 and ICC2 and within ICC2 ...................................................... 122
7 REFERENCES .................................................................................................................. 128

ACKNOWLEDGEMENTS .................................................................................................. 154
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA (reverse transcribed RNA)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>cysS</td>
<td>Gene coding for cysteinyl-tRNA synthetase</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IC</td>
<td>Immobilized cells</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRS</td>
<td>Man Rogosa and Sharpe medium</td>
</tr>
<tr>
<td>MRSC</td>
<td>MRS supplemented with 0.5% L-cysteine</td>
</tr>
<tr>
<td>NCC</td>
<td>Nestlé Culture Collection</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>purB</td>
<td>Gene coding for adenylosuccinate lyase</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR (real-time PCR)</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription real-time PCR</td>
</tr>
<tr>
<td>RAPD</td>
<td>Randomly amplified polymorphic DNA</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>USFA</td>
<td>Unsaturated fatty acid</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
</tbody>
</table>
Summary

Probiotics are defined as “live organisms which when administered in adequate amounts confer a health benefit on the host”. The delivery of live cultures to adequate levels at the target site in the host represents a challenging task in the production of probiotic food products. Probiotic bacteria, generally isolated from the gastrointestinal tract of humans, are fastidious microorganisms and susceptible to environmental stresses that occur during production, storage and transit through the gastrointestinal tract. Different strategies have been proposed to produce probiotics with enhanced stress resistance such as efficient screening methods to identify probiotic strains with intrinsic resistance to environmental stresses, genetic engineering, the pre-treatment of bacterial cells with sub-lethal stresses or the use of immobilized cells (IC) and continuous culture.

The dissertation consisted of three hypotheses: The first hypothesis claimed that \( B. \ longum \) NCC2705 cells produced as IC and continuous culture exhibits improved tolerance to environmental stresses which increases with culture age. The second hypothesis states that qRT-PCR can be used to quantify viable \( B. \ longum \) NCC2705 cells independently of morphological state. The third hypothesis is that spray drying can be used for preparation of bifidobacteria, and that random amplified polymorphic DNA (RAPD) technology can be used to identify the best survivor strain.

In a first approach, the probiotic strain \( Bifidobacterium \ longum \) NCC2705 was produced with (IC) technology in combination with continuous culture (Chapter 2). The production of immobilized \( B. \ longum \) NCC2705 was carried out in a two-stage fermentation system consisting of a first small reactor (R1) containing cells entrapped in gellan/xanthan gel beads for high cell productivity and a second larger reactor (R2) inoculated with bacteria produced from the first reactor. Reactor volumes of 360 ml (R1) and 1800 ml (R2) were chosen to yield a shorter
residence time in R1 than in R2, in order to obtain high cell productivity in R1 (high immobilized cell concentration at high dilution rates (2.25 h⁻¹)) followed by a final growth in R2 with longer residence time at a low dilution rate (0.2 h⁻¹). Two continuous cultures were produced for 20 days with and without glucose limitation. Both fermentations exhibited formation of macroscopic cell aggregates. Cells in the effluent from the second reactors showed enhanced tolerance to porcine bile salts and to aminoglycosidic antibiotics which were associated with modifications in the cell membrane composition. However, there was no significant difference in terms of culture age and heat resistance when compared to cells from free cell batch cultures.

Formation of large cell aggregates resulted in an underestimation of viable cell counts when measured by the traditional plate counting method. This complicated the characterization of physiological properties. Hence, we developed a new method to enumerate viable *B. longum* NCC2705 which is not influenced by the morphological state of the cells (Chapter 3). The new method was based on real time PCR technology and enabled the accurate quantification of cells in the aggregated state. To achieve this, we measured the level of a constitutively expressed housekeeping gene and calculated viable cell concentration using calibration curves from $7.0 \times 10^3$ to $4.7 \times 10^8$ CFU/ml. This quantification of IC samples containing aggregates resulted in up to 125-fold higher viable cell counts compared to traditional plate counts. The method was further used for analyzes of tolerance to bile salts of IC samples (see above).

In the last part of the thesis, a different strategy was evaluated to produce robust bifidobacteria. Instead of improving stress tolerance of one specific *B. longum* strain, we developed a high throughput screening method to identify bifidobacterial strains with intrinsic resistance to stresses occurring during spray drying and storage (Chapter 4). In this approach, twenty-two *B. longum* strains were mixed in separate batches and subjected to spray drying and storage experiments during two selection rounds. Unique identification of the twenty-two *B. longum* strains in the
mixed bacterial preparation was achieved using molecular-based methods. Within the mixed strain preparation, a robust strain was identified and stability was confirmed by performing single strain spray drying and storage tests. The selected strain exhibited a 250-fold better survival after 60 days of storage than the control strain. The method allowed faster screening of resistance of *B. longum* strains to spray drying and storage compared to traditional screening performed on individual strains.

In this thesis, IC and continuous cultures used to produce probiotic cells with improved stress resistance resulted in the formation of large cell aggregates. Auto-aggregation is a known phenotype in LAB and bifidobacteria and was associated with enhanced ability to adhere to host epithelial cells. Therefore it would be of interest to test adhesion ability of the cells produced with IC and continuous culture. Additionally, we designed and experimentally validated a high throughput screening method to select for probiotic strains with intrinsic resistance to stresses occurring during spray drying and storage. The same approach could be applied for selection of robust strains in response to other stress responses.
Zusammenfassung


Zusammenfassung

Um dennoch eine physiologische Analyse der immobilisierten Zellen durchzuführen, wurde im zweiten Teil der Dissertation eine neue Methode entwickelt, um die Anzahle lebender \textit{B. longum} NCC2705 Zellen unabhängig von ihrer Morphologie bestimmen zu können (Kapitel 3). Die Methode nutzt quantitative „echt Zeit“ Polymerasen Kettenreaktion (engl. qRT PCR) als ein Mittel, um die Kopienzahl eines sogenannten Haushaltsgens zu messen, um anhand einer Standardkurve, welche Zellkonzentrationen über eine Spannweite von \(7.0 \times 10^3\) bis \(4.7 \times 10^8\) Kolonien formende Einheiten pro ml abdeckt, auf die ursprüngliche Zellkonzentration in der Zellprobe zu schließen. Die Kopien eines Haushaltsgens wurden ausgewählt, weil zum einen die Anzahl der Kopien pro Zelle konstant ist, unabhängig von der Umgebung und des physiologischen Zustandes der Zelle, und zum andern weil die Kopien eines Gens (mRNA) instabil sind und kurz nach der Bildung wieder abgebaut werden. Ein konstantes Niveau der Kopienanzahl eines bestimmten Gens kann deshalb nur durch konstante Erneuerung und folglich nur von metabolisch aktiven, also lebenden Zellen erreicht werden. Eine gewisse Anzahl gemessener Kopien eines Haushaltsgens kann deshalb mit einer bestimmten Anzahl lebender Bakterien gleichgesetzt werden, was z.B. bei der stabileren DNA nicht der Fall ist, welche auch Stunden oder gar nach Tage nach dem Zelltod nachgewiesen werden kann.

Zusammenfassung

Zellklumpen, nachdem sie mit Gallensalzen oder Hitze behandelt wurden, zu berechnen und so die Toleranz von diesen Zellen gegenüber Gallensalzen oder Hitze zu testen (siehe oben).

Zusammenfassung

angezüchtet, sprühgetrocknet und gelagert und sein die Überlebensrate mit einem Kontrollstamm (NCC2916) verglichen wurde. Tatsächlich stellte sich heraus, dass Stamm NCC572 nach 60 Tagen Lagerung in Pulverform rund 250 Mal besser überlebte als der Kontrollstamm NCC2916. Die entwickelte Methode erlaubt die zeit- und kosteneffiziente Anwendung eines Auswahlprüfverfahrens, mit welcher probiotische Bakterienstämme mit inhärenter Stresstoleranz identifiziert werden können.

Die Methoden, welche in der vorliegenden Dissertation entwickelt wurden, können mannigfaltig angewendet werden. So kann z.B. das beschleunigte Auswahlprüfverfahren auf beliebig andere Konditionen angewendet werden. Ferner wäre es interessant, die verklumpten Bifidobakterien aus der kontinuierlichen Kultur mit immobilisierten Zellen, auf ihre Adhäsionsfähigkeit an menschlichen Epithelialzellen zu testen.
1. Introduction

1.1 Human gastrointestinal tract microbiota

The human gastrointestinal tract (GIT) is a complex system composed of different compartments: oral cavity, stomach, small intestine (duodenum, jejunum and ileum), and large intestine (caecum, colon and rectum) exhibiting very different environmental conditions. The intestinal epithelium has a combined surface area of 400 m$^2$ and provides ecological niches for the three domains of life: prokarya, archaea and eukarya. The GIT is one of the most dense microbial ecosystems on earth (Whitman et al., 1998), and its population number of up to $10^{14}$ cells exceeds that of the human cells by a factor of 10 (Backhed et al., 2004). Despite this tremendous number, the gut microbiota forms an exclusive community underlying a strict selection pressure. Gut microbes must be able to react rapidly to environmental changes, survive the harsh conditions during gastrointestinal transfer and during re-colonization of a new host, evade bacteriophages, and be capable of growth under GIT conditions to avoid disappearance (Ley et al., 2006). Gut inhabitants may also harbor a collection of enzymes for nutrient degradation and contain a cell-surface composition which might enable attachment to host epithelial cells (Ley et al., 2006).

The microbial colonization of the GIT varies with age and is characterized by temporary changes. The sterile GIT of newborns is colonized mainly by enterobacteria, bifidobacteria, and streptococci. The percental distribution depends on the environment, mode of birth, the diet and possibly on the host genetic background (Edwards and Parret 2002; Vaughan et al., 2002; Fanaro et al., 2003; Zoetendal et al., 2004). At older age, the microbial diversity increases to more than 1000 different subspecies of bifidobacteria, enterobacteria, streptococci, bacteroides, clostridia and cyanobacteria. However, a satisfying general representation of the ecosystem has not yet
been established because only a limited number of human gastrointestinal microflora has been analyzed and each subject harbors a unique microbial community (Rajilic-Stojanovic et al., 2007). The diversity of microflora develops with host maturation and gets more unstable with increasing age (Harmsen et al., 2000, Egert et al., 2006). Diversity of microflora is generally host specific and depends on factors such as digestion characteristics, gender, age and genotype, as well as on environmental factors like nutritional diet, ingestion of microorganisms and drugs (Zoetendal et al., 2006).

Host and gut microflora live in a symbiotic relationship; the host provides the microflora a nutrition-rich and protective habitat, while the microorganisms ferment non-digestible dietary substrates and indigenous mucus produced by epithelial cells, resulting in the production of short chain fatty acids (SCFA) which are absorbed by the host (Shanahan, 2002). The generated plus in dietary energy from fibre fermentation favors the adsorption of ions (Mg$^{2+}$, Ca$^{2+}$, Fe$^{2+/3+}$) in the caecum (Guarner et al., 2006). Additionally, the commensal flora provides a barrier against colonization of exogenous and pathogenic bacteria by outcompeting them for nutrients and binding sites (Guarner et al., 2006) and by producing antimicrobials such as bacteriocins inhibiting (a.o.) growth of pathogens (Shanahan, 2002). Besides protection, the gut microbes are involved in developmental and immunological processes of the host by modulating immune responses and influencing proliferation and differentiation of epithelial cells (Hooper et al., 2001; Falk et al., 1998, Guarner and Magdalena, 2003). Endogenous bacteria are also involved in the production of vitamin B$_{12}$, vitamin K, biotin, folic acid, in the synthesis of amino acids from ammonium and urea, and in the inactivation of dietary carcinogens (Wollowski et al., 2001, Hooper et al., 2002). However, the complex microflora with its huge diversity of different species and subspecies is a major obstacle in the prediction of specific bacterial function within the GIT.
The development of molecular techniques allowed the exploration of the human microflora and revealed new insights during the last decade. In the future, advanced molecular tools will confer on understanding of how single or groups of bacteria interact with the host, thereby providing new leads for additional investigations. One of the key players in the transient microflora of newborns and also for the development of probiotic products is the genus *Bifidobacterium*, displaying an appearance ranging from 40 – 90 % of total bacteria counts in the intestine of infants (Harmsen *et al.*, 2000).

### 1.2 Bifidobacterium

#### 1.2.1 General characteristics

Bifidobacteria were first isolated from faeces of breast-fed infants by Tissier in 1906. They are among the first species to colonize the sterile GIT of newborns. In adults, *Bifidobacterium* is the third most common genus, after bacteroides and eubacteria (Bezkorovainy, 2001). Bifidobacteria are Gram-positive, heterofermentative, non-motile, non-sporulating bacteria. They vary in size and exhibit rods of various shapes, often in ‘V’ or ‘Y’ patterns (Latin *bifidus*: cleft, divided). Although considered strictly anaerobic, some strains exhibited poor growth in presence of oxygen (Simpson *et al.*, 2005). Most strains isolated from humans grow optimally at temperatures of 36 – 38 °C. Bifidobacteria are often included in the group of LAB due to their metabolic capacities and the sharing of ecological niches (Klein *et al.*, 1998). However, phylogenetically the genus *Bifidobacterium* belongs to the family of actinomycetaceae, a family including *Corynebacterium*, *Mycobacterium*, and *Streptomyces*. Actinomycetaceae share the common feature of having a high G+C content ranging from 42 to 67 %, which is sufficiently higher to differentiate them from lactic acid bacteria (Biavati *et al.*, 2000). The genus
Bifidobacterium is relatively small with ca. 32 identified species to date, mainly of human or animal origin and has a low level of phylogenetic and genomic diversity (Ventura et al., 2006). The availability of genome sequences of microorganisms from the human GIT and progress in molecular tools will lead to a better understanding of the behavior and survival of these bacteria in the GIT. Up to today, the two B. longum strains NCC2705 (Schell et al., 2002) and DOJ10A and two strains of B. longum subsp. infantis (B. longum subsp. infantis ATCC55813 and ATCC15697) have been sequenced (Lee et al., 2008; Sela et al., 2008).

1.2.2 Metabolism of Bifidobacteria

Bifidobacteria can utilize a large variety of carbohydrate sources. Utilization of complex carbohydrates from dietary compounds confers a competitive advantage for bifidobacteria in the lower GIT (Hooper et al., 1999; Klijn et al., 2005). Bifidobacteria metabolize carbohydrates exclusively and characteristically in the so-called “bifido-shunt” via the key enzyme fructose-6-P phosphoketolase (F6PPK). This enzyme converts hexoses mainly to lactic and acetic acid (Scardovi and Tovatelli, 1965). However, sugar availability and consumption rate affects production of other metabolites, such as formic acid and ethanol, thereby altering the molecular ratio of these acids (van der Meulen et al., 2006).

Progressive genome sequencing technologies have disclosed an insight in encoded enzymes involved in metabolic pathways. Genome annotations of bifidobacteria revealed that 8% of the genes code for enzymes involved in the carbohydrate metabolism which exceeds the percentage of other GIT inhabitants such as Enterococcus faecium and Echerichia coli or allochthonous Lactococcus lactis (Ventura et al., 2007). Sequence in combination with physiological analyzes confirmed that bifidobacteria utilize a broad range of indigestible polysaccharides like hog gastric
mucin, pectin, plant oligosaccharides and fructo-oligosaccharides (Schell et al., 2002; MacConaill et al., 2003, Backhed et al., 2004, Matsuki et al., 2004; Parche et al., 2007). Taken together, bifidobacteria have a unique potential to metabolize indigestible components, which helps to prolong residence time in the competitive environment of the human gut.

1.2.3 Role of Bifidobacteria in the GIT

Each compartment of the GIT is a habitat for a specific bacterial community. Bifidobacteria can be found all along the GIT and the main species present in humans are *B. adolescentis*, *B. bifidum*, *B. infantis*, *B. breve* and *B. longum* (Crociani et al., 1996; Marteau et al., 2001; Nielsen et al., 2003; Champagne et al., 2005). The preferred location of bifidobacteria inside the GIT cannot be easily determined since numbers and species vary between individuals (Nielsen et al., 2003). Furthermore, the majority of studies on human microbiota comes from analyzes of fecal contents and are thus limited in reflecting the distribution of species in the different habitats of the GIT (Tannock 1999; Marteau et al., 2001). Bifidobacteria can be autochthonous (found where they grew) or allochthonous (found at places other than where they grew). The first colonizers of the sterile GIT of newborns are mostly strains that reside autochthonously after colonization (Vaughan et al., 2005).

In adults, species-diversity of microbiota increases and percentage of bifidobacteria decreases with age depending on the health of the individuals (Hopkins and MacFarlane, 2002; Vaughan et al., 2002; Hebtuerne et al., 2003; Woodmansey et al., 2004). This decrease was associated with the fact that allochthonous bifidobacteria do not naturally occur in food bifidobacteria but remains subject of discussions. However, bifidobacteria are added to food for their claimed health benefits and their ingestion results in a temporary (elevated) appearance in the GIT with average
retention time believed to be less than a week (Kullen et al., 1996, Fukushima et al., 1998), while other studies claim it to be more than one week (Alander et al., 2001, Ouwehand et al., 2004, Bartosch et al., 2005, Su et al., 2005). Ouwehand and coworkers summarized in their review in 2004 that several factors to affect the residence time of allochtonous bacteria such as duration of the consumption, amounts of the administered doses, and the methods of detection used.

1.2.4 Transcriptomics of *Bifidobacterium longum* NCC2705

The transcriptome is defined as the set of all messenger RNA (mRNA) molecules, also called "transcripts," produced in a population of cells. Since it includes all mRNA transcripts in the cell, the transcriptome reflects the genes that are being actively expressed. Therefore, this relatively new molecular tool can give insights into global cellular mechanisms under different environmental and/or growing conditions. However, transcriptome analyzes require the availability of DNA based arrays, thereby complete genome sequence of the tested organism. The strain *B. longum* NCC2705 was the first completely sequenced genome within the genus *Bifidobacterium* (Schell et al., 2002) and a microarray covering its whole genome is available at Nestlé Research Center. Transcriptomic analyzes of this strain have been used to characterize metabolism and growth on various sugars (Parche et al., 2007) as well as cellular response to environmental stresses. The exposure of *B. longum* NCC 2705 cells to oxygen and starvation induced expression of genes that are known to participate to the common stress response in other bacteria, whereas other up-regulated genes still remain unidentified (Klijn et al., 2005). In 2007, Rezzonico and co-workers tested the gene expression patterns of *B. longum* NCC 2705 after exposure to different heat periods and reported a 46 % of differentially expressed genes, including common bacterial stress-responses such as the induction of chaperones and the down-
regulation of the translation, cell division and chromosome-partitioning machineries. In a recent study, the effect of subinhibitory concentrations of bile on the expression levels of three different bifidobacterial strains including \textit{B. longum} NCC2705 were tested and lead to the identification of a new multidrug resistance transporter in strain NCC2705 (BL0902) conferring increased resistance to bile salts (Gueimonde \textit{et al.}, 2009).

These results emphasize the importance of transcriptome analyzes which enable new insights into general cellular mechanisms and may help to design probiotic strains with enhanced functional properties (Corcoran \textit{et al.}, 2008). Moreover, transcriptomics may also support the analysis of complex physiological or morphological changes of bacteria as a result of changing environments.

\section*{1.3 Probiotic concept}

In 1907 the Russian scientist Elie Metchnikoff was the first to mention the beneficial effects of bacteria in his book “The prolongation of life” and presented the concept of “replacement of harmful microbes by useful microbes”. In 1906, Henry Tissier claimed that bifidobacteria are the dominant species in the microflora of breast-fed infants and recommended the administration of bifidobacteria to out-compete the putrefactive bacteria in infants suffering diarrhea. The term “probiotic” (Greek: pro life) was introduced by Lilly and Stillwell (1965) and after several redefinitions, the Joint Food and Agricultural Organization and World Health Organization (FAO/WHO) working group defined the term “probiotic” in 2001 as “live microorganisms that when administered in adequate amounts confer a health benefit on the host”. Probiotic bacteria exert beneficial effects like inhibition of colonization of potential pathogens of the gut, immunomodulation, alleviation of constipation, reduction of problems associated with
assimilation of lactose, and alleviation of antibiotic-associated diarrhea (reviewed by DeVrese and Schrezenmeir, 2008; see “1.4 Health promoting properties related to probiotics”).

Growing scientific interest in probiotic bacteria is reflected in the increasing number of scientific publications on the subject (Figure 1).

Figure 1  Number of publications obtained with the key word “probiotic” in the Pubmed database as a function of time. Dashed bar is an extrapolated value based on number of publication obtained after 6 months in 2009.

Nowadays, the prospering probiotic food market in Western Europe generates more than 1.4 billion Euros, one billion of which is exclusively from yogurt and desserts. Annual growth of sales is forecasted to be 7 – 8 % over the next five years (Saxelin, 2008). Besides dairy products, probiotics are also administered in fruit juices, berry soups, ice cream, soy- and cereal-based fermented products as well as cheese. Probiotic foods are manufactured either by adding the desired strains simultaneously with the starter culture in the fermentation tank or by running the fermentation in a separate reaction and adding the probiotics afterwards in frozen liquid or formulated state (Champagne et al., 2005). The increase of probiotic consumption raises a
demand by the food industry for more scientific research to develop new methods to improve quality, safety, and functionality of probiotics.

1.4 Health promoting effects related to probiotics

Potential probiotic strains are screened for their health promoting qualities based on the following established selection criteria (reviewed by DeVrese and Schrezenmeir, 2008)

- Origin from the intestinal tract of healthy persons, as such microorganisms are regarded safe for humans and best adapted to the ecosystem of the gut
- Safe for humans, free of pathogenic and toxic effects
- Tolerance and resistance to gastrointestinal conditions and digestive enzymes enabling the survival during the passage through stomach and upper intestinal tract eventually resulting in health-promoting effects in the bowel
- Possess properties associated with a (positive) influence on the intestinal flora, like adhesion to intestinal epithelial cells, survival and reproducing capacity in the human large intestine, or production of antimicrobial substances

The most common strains used in probiotic food products are either lactobacilli or bifidobacteria. Other strains exerting probiotic effects belong to the genus of Propionibacterium which is not typical for human microbiota but strains of this genus were shown to produce vitamin B12, and bacteriocins in addition to organic acetic and propionic acids from sugar metabolism. Furthermore, yeasts from the genus of Saccharomyces have been show to exhibit antagonistic effects against enteric pathogens (Klein et al., 1993) or are used as preventive and therapeutic agents for the treatment of a variety of diarrheal disease.
The beneficial effects of strains of bifidobacteria have been attested \textit{in vitro} in numerous studies. However, probiotic properties have to be confirmed also \textit{in vivo} before claiming them as probiotic strains (Reid, 2008) and therefore a number of clinical studies on the therapeutic effects of supplementation of bifidobacterial strains in mouse models and humans have been conducted in the recent years as illustrated in Table 1.
Table 1  A selection of studies with beneficial effects on humans or mice of products containing bifidobacteria

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Benefit</th>
<th>Host(^a)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. longum</em> B6 and ATCC 15708</td>
<td>Reduction of lactose intolerance</td>
<td>Humans (15)</td>
<td>Jiang et al., 1996</td>
</tr>
<tr>
<td><em>B. infantis</em></td>
<td>Reduced risk of necrotizing enterocolitis (NEC)</td>
<td>Rats, neonates</td>
<td>Caplan et al., 1999</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>Protection against <em>S. enteridis</em> ssp. <em>typhimurium</em></td>
<td>Mouse, healthy and gnotobiotic</td>
<td>Silva et al., 1999</td>
</tr>
<tr>
<td><em>B. lactis</em> HN019</td>
<td>Enhance natural immunity in healthy elderly subjects</td>
<td>Human (25)</td>
<td>Arunachalam et al., 2000</td>
</tr>
<tr>
<td><em>B. lactis</em> Bb-12</td>
<td>Decrease of extent and severity of atopic eczema</td>
<td>Human, infants (27)</td>
<td>Isolauri et al., 2000</td>
</tr>
<tr>
<td>Strain mix: <em>Lactobacillus</em> 4, <em>Bifidobacterium</em> 3, and 1 <em>Streptococcus</em></td>
<td>Prevent flare-ups of chronic pouchitis</td>
<td>Human (40)</td>
<td>Gionchetti et al., 2003</td>
</tr>
<tr>
<td><em>B. lactis</em> Bb 12</td>
<td>Protective effect against acute diarrhea</td>
<td>Humans, children (90)</td>
<td>Choraqui et al., 2004</td>
</tr>
<tr>
<td><em>B. pseudocatenulatum</em> DSM 20439, <em>B. breve</em></td>
<td>Inhibit Shiga toxin-production of <em>E. coli</em> (STEC) 0157:H7</td>
<td>Mice</td>
<td>Ashara et al., 2004</td>
</tr>
<tr>
<td>Strain mix: <em>Lb. confusus</em>, <em>Lb. fermentum</em>, <em>Lb. plantarum</em>, <em>B. infantis</em> PL9506</td>
<td>Suppression of Th2 cytokines during antigen sensitization</td>
<td>Mice</td>
<td>Lee et al., 2004</td>
</tr>
<tr>
<td>Strain mix: <em>Lb. gasseri</em> PA 16/8, <em>B. longum</em> SP 07/3, <em>B. bifidum</em> MF 20/5</td>
<td>Reduced severity and duration of cold episodes</td>
<td>Humans (479)</td>
<td>de Vrese et al., 2005</td>
</tr>
<tr>
<td>Strain mix: <em>B. infantis</em>, <em>S. thermophilus</em>, <em>B. bifidus</em></td>
<td>Reduction of incidences and severity of necrotizing enterocolitis (NEC)</td>
<td>Human, neonates (145)</td>
<td>Bin-Nun et al., 2005</td>
</tr>
<tr>
<td><em>B infantis</em> 35624</td>
<td>Alleviates symptoms of irritable bowel syndrome (IBS)</td>
<td>Humans with IBS (77)</td>
<td>O'Mahony et al., 2005</td>
</tr>
<tr>
<td><em>B. lactis</em> (Bb-12)</td>
<td>Fewer and shorter episodes of diarrhea</td>
<td>Humans (200)</td>
<td>Weizman et al., 2005</td>
</tr>
<tr>
<td><em>B. thermacidophilum</em> RBL 71</td>
<td>Reduced severity of <em>E. coli</em> O157:H7 infection</td>
<td>Mice</td>
<td>Gagnon et al., 2006</td>
</tr>
<tr>
<td><em>B. lactis</em> HN019</td>
<td>Enhanced aspects in cellular immunity in the elderly</td>
<td>Humans, elderly (30)</td>
<td>Ahmed et al., 2007</td>
</tr>
<tr>
<td>Strain mix: <em>Lb. acidophilus</em> NCFM, <em>B. animalis</em> ssp. <em>lactis</em> Bi-07</td>
<td>Reduced fever, rhinorrhea, and cough incidence and duration and antibiotic prescription incidence</td>
<td>Humans, children (326)</td>
<td>Leyer et al., 2009</td>
</tr>
<tr>
<td>Strain mix: <em>Lb. rhamnosus</em> GG, <em>B. lactis</em> Bb-12</td>
<td>Reduced risk of early acute otitis media and recurrent respiratory infections during the first year of life</td>
<td>Humans, children (81)</td>
<td>Rautava et al., 2009</td>
</tr>
</tbody>
</table>

\(^a\) in brackets: number of persons tested
The particular molecular mechanisms underlying most of the probiotic effects of bifidobacteria are still not completely understood (Leahy et al., 2005). Bifidobacterial strains exhibited antimicrobial activities against other gut inhabitants which are attributed to production of bacteriocins or organic acids that lower the colonic pH thereby inhibiting acid sensitive pathogenic bacteria (Klijn et al., 2005; Cheikhyoussef et al., 2008). Bifidobacteria can modulate the immune response by regulating the production of anti- and proinflammatory cytokines in peripheral blood mononuclear cells of humans (Medina et al., 2007). Strains of bifidobacteria were shown to produce linolenic acid in vitro (Coakley et al., 2009) or vitamins (Tamine et al., 1995), both substances that can contribute a health benefit to the host. Genome analyzes revealed the complete pathway for production of folic acid, thiamine, and nicotinate in *B. longum* NCC2705 (Schell et al., 2002). Metabolic activity may moreover reduce serum cholesterol level through bile salt hydrolase activity (De Smet et al., 1998).

### 1.5 Potential risks associated with probiotics

There are three theoretical concerns regarding the safety of probiotics (Snydman, 2008).

1. They may lead to disease, such as bacteremia or endocarditis.
2. Toxic or metabolic effects on the gastrointestinal tract
3. Transfer of antibiotic resistance in the gastrointestinal flora

In rare cases, *Bifidobacterium* species were shown to be involved in human dental caries, pulmonary infections, bacteremia, abscesses, and blood stream infections which were not related to consumption of probiotic products (Green, 1978; Gasser, 1994; Saarela et al., 2002; Modesto et al., 2006). A tetracycline resistance gene (*tet(W)*) was reported on the chromosome of *B. lactis* DSM 10140 (Kastner et al., 2006) which is problematic due to possible horizontal gene transfer.
to pathogens in the gut. In patients with short small bowel syndrome, deconjugated bile acid metabolites produced by bifidobacteria can accumulate leading to malabsorption of food nutrients (Snydman, 2008). However, bifidobacteria and lactobacilli (with exception of \textit{L. rhamnosus}) used in probiotic food products are “generally accepted as safe” (GRAS) by the Food and Drug Administration of the USA and classified as “class I” (absolutely safe) in Germany. Furthermore, there is no evidence for a higher risk of bacteremia or fungemia due to the ingestion of probiotic products in comparison with conventional fermented food products (Snydman, 2008; Salminen \textit{et al.}, 2002).

1.6 Technological requirements of probiotic bacteria

In order to provide a therapeutic effect to the consumer probiotic food products should contain a critical concentration of viable bacteria per gram of product (Tamime \textit{et al.}, 1995). Studies with non-viable cells or cell-components of probiotic strains also showed positive effects on health or immunomodulatory effect (Salminen \textit{et al.}, 1999; Lammers \textit{et al.}, 2003). However, the average recommended level of viable probiotic bacteria is suggested between $10^6$ to $10^7$ CFU per gram of product at the time of consumption (Talkamar \textit{et al.}, 2004; Ishibashi and Shimamura, 1993). Additionally, the probiotic food product should be regularly consumed in sufficient quantities to deliver the relevant “dose” of live bacteria to the gut. Nevertheless, increasing knowledge of strain survival during GIT transit and strain specific therapeutic effect will probably modify these numbers in the near future (Champagne \textit{et al.}, 2005). The achievement of sufficient viable probiotic cells at the time of consumption and at target site remains a challenge for the food industry.
The preparation of a critical level of viable cells in probiotic food products and activity at the target site depends on conditions during fermentation, downstream processing, storage and during gastrointestinal transit (Figure 2). Exposure of microorganisms to different stresses decreases bacterial viability and causes large fluctuations in viable cell counts of probiotic products. In fact, numerous probiotic food products failed to meet the minimal level of viable bacteria in probiotic food products (Masco et al., 2005, Huys et al., 2006). The selection criteria of probiotic candidate strains are thus mainly focused on their technological properties, which means that a lot of strains with promising health properties are probably missed (Lacroix and Yildirim, 2007).

**Figure 2** Main factors affecting the viability of probiotics from production to the gastrointestinal tract (Lacroix and Yildirim, 2007).

Economic reasons raise the demand of food industries for new technologies that enable high viable cell yield at large scale production. Major drawbacks in propagation and scale-up process are sensitivity to oxygen and acidic conditions of numerous strains of intestinal origin. Technologies which ensure probiotic activity and stability in food products are thus of highest
interest. Improved survival of strains during manufacturing may enable production of technologically less favorable candidate strains but with more relevant probiotic properties, consequently resulting in higher product efficacy. Therefore, the selection of adequate strains and the improvement of technologies for production of probiotics is crucial in the development of new probiotic food products.

1.7 Technologies to improve cell viability and yields

1.7.1 Production technologies

1.7.1.1 Optimization of growth medium

Probiotic strains are preferentially added to dairy products after fermentation because the fast growth and acidification rate of typical starter cultures are disadvantageous for probiotic culture development and maintenance (Champagne et al., 2005). Probiotics are delivered as frozen liquid or as dry state cultures produced with freeze- or spray drying technology (see “Stabilization of probiotics”). The cultivation conditions prior to freezing or drying have an impact on growth, culture stability and activity as well as on drying and subsequent storage (Reilly and Gilliland 1999; Desmond et al., 2002; Carvalho et al., 2004). Modulation of the culture conditions can therefore improve stability and activity of probiotics in food products.

Traditional large scale production of cells is performed in large batch fermentations. Approaches to enhance yields in biomass and enhance cell stability have ranged from designer growth medium to alternative fermentation technologies. In research laboratories, expensive media such as MRS broth (de Man, Rogosa, and Sharpe, 1960) is commonly used for cultivation of LAB or bifidobacteria. Biomass production from ultra-filtered skim milk with different protein concentrations or supplementation of milk with nitrogenous substrates such as whey and casein
fractions from human or cow milk resulted in higher cell counts of bifidobacterial strains compared to growth in skim milk only (Ventling and Mistry, 1993; Petschow and Talbott, 1990). In contrast, bifidobacteria grown in soymilk or animal-product-free vegetable medium (based on soy peptone, glucose and yeast extract (YE)) displayed lower yields or lower viability during storage conditions due to the low buffer capacity of vegetable medium (Heenan et al., 2002, Shimakava et al., 2003). Recently, a study showed a significantly increased yield of cell after 24 h growth of a *B. animalis* strains or a *B. longum* strain in modified vegetable medium compared to standard MRS medium (Mättö et al., 2006). Growth medium for bifidobacteria is generally supplemented with redox-reducing compounds such as cysteine to improve growth (Doleyres and Lacroix, 2005). However, the growth promoting properties of such supplements lose the effect when the disulfide bonds are reduced, for example by performic acid oxidation or reduction-alkylation (Poch and Bezkorovainy, 1991; Ibrahim et al., 1994). Summarized, higher biomass yields of bifidobacteria can be achieved by modulation of the medium components and opens possibilities to develop low cost production methods.

### 1.7.1.2 Stress adaptation

The capacity of microorganisms to adapt to adverse environments is associated with the expression of stress proteins and the development of cross resistances to numerous stresses (Ross *et al.*, 2005; Lindner *et al.*, 2006). Besides medium composition, growth conditions like pH, fermentation time or addition of protectants can induce cell responses that particularly affect stability during downstream processing, storage, and transit through stomach and duodenum. The enhanced tolerance to environmental stresses of cells from stationary growth phase compared to exponentially growing cells is well established (Kolter, 1999) and reflects the bacterial ability for
rapid adaptation to changing conditions. For example, improved tolerances to acidic conditions in \( L.\ acidophilus \) could be linked to an over-expression of a \( \text{F}_1\text{F}_0\text{-ATPase} \), an enzyme which is actively involved in proton extrusion out of the cytoplasm (Kullen and Klaenhammer, 1999). Exposure of oxygen tolerant \( B.\ longum \) strains to oxygen caused an induction of an Osp-protein expression as well as changes in the bacterial membrane compositions such as an increase of short- and cyclopropane fatty acids in the cell membrane to protect the cell from damage (Ahn et al., 2001).

Using a proteomic approach, several up-regulated stress associated enzymes have been identified in \( B.\ longum \) cells upon exposure to bile salts (Sanchez et al., 2005; Savijoki et al., 2005) or high temperatures (Savijoki et al., 2005). These included general stress-related chaperones, proteins involved in transcription and translation and some proteins from different metabolic pathways. In a recent study of global gene expression, stress response upon heat treatment of \( B.\ longum \) NCC2705 showed that 46 % of genes exhibited altered gene-expression. These included the classical heat shock stimulon including chaperones DnaK, DnaJ or GroEL/ES which assure correct folding of proteins (Sugimoto et al., 2008) and enzymes involved in repression of cell division process or proteases to digest heat-induced misfolded proteins (Rezzonico et al., 2007). Proteomic analyzes of \( B.\ longum \) and \( B.\ adolescentis \) upon bile treatment showed higher levels of chaperones and enzymes involved in fatty acid synthesis, resulting in changes in membrane fatty acid composition (Sanchez et al., 2007). These changes may favor tolerance to bile salts by protecting cells from passive diffusion of bile salt into cytoplasm (Begley et al., 2005, Ruiz et al., 2007). A model of a number of adaptation mechanisms to bile salt stress of bifidobacteria was recently developed by Sanchez et al. (2008) based on data acquired from proteomic analyzes (Figure 3).
The ability of microbes to adapt to stress conditions resulting in improved survival has been widely reported and exploited in yogurt starter culture *Streptococcus thermophilus* or probiotic cultures (Schmidt and Zink 2000; Wouters et al., 1999; Saarela et al., 2004). Bifidobacteria treated with various stress conditions such as low pH, heat or combined stresses resulted in increased stress responses to homologous stresses or cross-protection (Saarela et al., 2004). Increasing sublethal treatment with NaCl, bile salts or heat exposure enhanced survival of several bifidobacterial strains to lethal heat stress or freeze-thawing (Schmidt and Zink, 2000). Starvation of *B. longum* for 30 or 60 min or exposure of *B. lactis* to pH 5.2 under starvation conditions increased survival to prolonged cold storage in growth medium and lethal acidic conditions, respectively (Maus and Ingham, 2003). However, cellular responses to pre-conditioning and the subsequent tolerance to lethal stress depend on the sublethal stress (duration, severity, combination) and the particular strain used (Simpson et al., 2005). The molecular mechanisms involved in stress resistance have still to be clarified in detail with the help of global stress response analyzes such as proteomics and transcriptomics (Sanchez et al., 2008). Consequently, optimization of sublethal treatment has to be assessed strain by strain which is time consuming and tedious. Recently, a system has been developed in our laboratory using continuous culture in combination with a two-stage fermentation system which allows the efficient screening of a number of sublethal stresses under controlled conditions (Mozzetti, 2009). Nevertheless, bacterial stress response is complex and the number of molecular mechanisms rendering stress resistance remains to be explored. An alternative to sublethal stress adaptation of probiotics is the use of immobilized cell technology. In previous studies it was shown that immobilized cells during continuous culture can be used to produce probiotic strains with improved functional and technological properties (Lacroix and Yildirim, 2007).
Figure 3 A model of main physiological mechanisms involved in bile salt tolerance of bifidobacteria. Conjugated bile acids/salts diffuse to the bifidobacterial cytoplasm (1), and are cleaved by the bile salt hydrolase (BSH; bile adaptation and acid adaptation protein) and rendering one amino acid (glycine or taurine) and one deconjugated bile acid moiety (2). Unconjugated bile salts can also enter the cytoplasm by passive diffusion (3), being deprotonated at the slightly acid pH of the cytoplasm (4). Ionized bile acids are non-permeable, and must be excreted by the action of certain transporters, such as the cholate transporter “Ctr” of B. longum (5) (Price et al., 2006). In addition, the process of tolerance to bile salts is associated with an increase in the synthesis of molecular chaperones (6) and a decrease in the synthesis of long-chain-fatty-acid CoA ligase (bile response and adaptation protein) together with a shift in the fatty acid composition (7). Bile acid deprotonation cause cytoplasm acidification, which is counteracted with mechanisms such as ammonia production from glutamine deamination (8) or proton pumping by the F$_1$F$_0$-ATPase (acid response and bile adaptation protein) (9). The amounts of ATP needed for feeding these mechanisms are provided through the bifid shunt (10) (adapted from Sanchez et al., 2008).

Abbreviations: AA: amino acid; BCAA: branched chain amino acid; Ctr: cholate transporter
1.7.1.3 Immobilized cell (IC) technology in continuous cultures

Fermentation technologies, such as continuous culture and immobilized cell systems, are not widely established in food industries, yet they have potential for enhancing the performance of fastidious probiotic organisms. These technologies might be employed to develop strains with improved physiology and functionality when introduced into the gut and to enlarge the range of commercially available probiotics, as well as expanding product applications (Lacroix and Yildirim, 2007). In continuous cultures, bacteria grow in a bioreactor with continuous feed of fresh medium and removal of fermented broth at a given dilution rate. Steady conditions in continuous cultures allow analysis of metabolism, growth rate and gene expression of bacteria under constant conditions during long time period (Hoskisson and Hobbs, 2005). Continuous culture of *B. longum* SH2 exhibited increased volumetric biomass productivity compared to traditional batch cultures (Kim *et al.*, 2003). Recently, a *B. longum* NCC2705 culture grown in prolonged chemostat culture for 200 h showed metabolic and transcriptomic stability throughout entire culture time enabling to apply these cells in a new method to screen for sublethal treatments (Mozzetti, 2009). Nevertheless, a substantial drawback of continuous cultures is the increased susceptibility to contamination. The permanent feeding of fresh medium must be maintained at dilution rates lower than the maximum specific growth rate to prevent wash out of active biomass. Low dilution rate on the other hand increases probability of a competitive contaminant strain to establish in the bioreactor.

1.7.1.3.1 Principle of cell immobilization

The principle of immobilized cell fermentation is based on the retention of microorganisms in a discrete location of the fermentation to yield high biomass and/or to protect bacteria from an
antagonistic environment. Several immobilization techniques have been applied in dairy fermentations to produce starter or probiotic cultures and metabolites, including attachment or adsorption to a preformed carrier, membrane entrapment, microencapsulation and physical entrapment in polymeric beads (Lacroix et al., 2005).

1.7.1.3.2 Techniques of cell immobilization

A promising technology using immobilized cells to produce probiotic cultures is the entrapment of microorganisms in a food-grade porous polymeric matrix (Lacroix et al., 2005; Lacroix and Yildirim, 2007). Cell entrapment is assessed by inoculating liquid polymeric matrix with fresh cell culture followed by droplet formation of the polymer-cell mixture using extrusion or emulsification. Spherical polymer gel beads of diameters between 0.3 – 3 mm are formed either by thermal gelation (κ-carrageenan, locust bean gum (LSB), gellan, agarose, gelatin) or ionotropic hardening (alginate, chitosan). After gelation, polymeric beads with the entrapped viable cells are incubated in growth medium to colonize the beads (Lacroix et al., 2005).

When immobilized cells are incubated in growth medium, diffusion limitations occurring in gel beads for both substrates and inhibitory products, mainly lactic and acetic acid in the case of LAB, confer a more favorable environment for cell growth close to the bead surface than at the bead center (Arnaud et al., 1992; Masson et al., 1994; Lamboley et al., 1997; Cachon et al., 1998; Doleyres et al., 2002a). The very high density of cells at the bead surface can be compared to bacterial biofilm structures (Lamboley et al., 1997). Colonized polymeric gel-beads incubated in stirred reactors release large numbers of cells from their peripheral layers to the growth medium upon pressure due to cell expansion, collision and shearing forces (Sodini et al., 1997; Lamboley et al., 1999).
1.7.1.3.3 Physiological changes of immobilized cells

Several studies have shown that immobilized and released bacteria exhibit changes in growth, morphology and physiology compared with cells produced in conventional free-cell cultures for LAB and probiotics (Lacroix and Yildirim, 2007). For instance, immobilized *Lb. plantarum* showed changes in cell morphology from rod to coccoid shape along with a shift from homo- to heterofermentative metabolism (Krishnan *et al.*, 2001). *Saccharomyces cerevisiae* and *Acetobacter aceti* exhibited enhanced tolerance to ethanol and acetic acid upon cell immobilization (Krisch and Szajani, 1997). Similar effects were reported for immobilized *Escherichia coli* to phenol and antibiotics (Heipieper, *et al.*, 1991; Jouenne *et al.*, 1994) and *Lactococcus* and *Leuconostoc* strains to quaternary ammonium sanitizers (Trauth *et al.*, 2001). These alterations in cell physiology or metabolism may have different origins. The biofilm-like growth on bead surface induces changes in cell response. Natural biofilms comprise of a mixture of bacteria, proteins, nucleotides and exopolysaccharides which offers protection from restrictional environment in the host (Stanley and Lazazzera, 2004). Biofilm-embedded cells exhibit resistance to environmental stresses like high temperatures, low pH, osmolarity and biocides including antibiotics due to metabolic slowdown and physical protection by the cell community (Costerton *et al.*, 1999; Fux *et al.*, 2005). When biofilms reach a certain size, complex structures are formed composed of pillars of water channels to allow nutrient influx and waste efflux (Davey and O’Toole, 2000). Such a complex community requires some kind of inter-bacterial communication known as quorum sensing. Bacteria accumulate specific compounds (peptides, bacteriocins) to a certain threshold, thereby controlling expression of various genes involved in regulation and adaptation to the exterior environment within the biofilm (Stanley and Lazazzera, 2004). Although gel beads are artificially produced, the high cell density on the bead surface are comparable to biofilm environment and could induce quorum-
sensing responses, leading to improvement in physiological and technological characteristics that are important for industrial applications of probiotics. The adaptation process might provide a competitive advantage for the population, more effective adherence properties and rapid responses to changing environmental conditions when introduced to a host (Sturme et al., 2002). Alternatively, changes in cell morphology and physiology can be triggered by non-specific stress adaptation during maturation of cells caused by the steep gradients of pH, nutrients and inhibitors (organic acids) dominating the interior of the colonized gel beads (Doleyres et al., 2002b).

1.7.1.3.4 Advantages of continuous IC cultures

Continuous IC systems are usually processed at high dilution rates because physical retention of cells prevents wash-out of active biomass and provide efficient biomass production. High dilution rate ensures high substrate supply for cell growth and metabolism and rapid removal of metabolites to prevent product inhibition, and diminishes risk of contamination. Continuous culture with IC might also favor selection of stress tolerant subpopulations during prolonged fermentation time (Doleyres et al., 2004a). Other advantages over free cell fermentations are the reuse of biocatalysts, less sensitivity to contamination and bacteriophage attack, enhanced plasmid stability, and physical and chemical protection (Doleyres and Lacroix, 2005). Immobilized starter or probiotic bacteria have been produced using repeated batch or continuous culture and exhibited various benefits compared to traditional batch free cell cultures (Table 2). The complex cell response upon growth in polymeric gel beads remains to be elucidated. Further knowledge about the mechanism responsible for the stress adaptation on a physiological and global cell response level (transcriptomics, proteomics, metabolomics) may lead to the production of probiotic strains with enhanced technological and biological properties during cell production, gut transition and colonization in the human GIT (Lacroix and Yildirim, 2007).
Table 2  
Fermentation systems with immobilized LAB or bifidobacteria and the corresponding bacterial phenotype

<table>
<thead>
<tr>
<th>Fermentation system/ immobilization matrix/ growth medium</th>
<th>Species</th>
<th>Benefit of IC</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1-stage continuous/ polyacrylamid lattice/ Milk | *Lb. casei* | Investigate effect of IC on cell metabolism  
- Stable production of metabolites  
- Bead stability | Diviès and Siess, 1976 |
| 2-stage continuous/ fed batch/ alginate/ milk or cream | *Lb. bulgaricus*  
*St. thermophilus*  
*St. diacetylactis; St. lactis; St. cremoris Lc. Lactis; Lc. diacetylactis* | Yogurt/Cheese production and cream fermentation  
- Constant yogurt production, small volume  
- Cheese with constant properties, small volume  
- Cream with improved storage properties | Prévost et al., 1985;  
Prévost and Diviès, 1987 and 1992 |
| 1-stage continuous/ κ-carrageenan, locust soy bean (LSB)/ reconstituted skim milk, yeast extract (YE) | *B. infantis* | Testing applicability of IC in milk fermentation  
- high bead stability  
- high biomass/cell volumetric productivity | Ouellette et al., 1994 |
| 2-stage continuous/ κ-carrageenan, LSB/ whey permeate, YE | *Lb. helveticus* | Kinetic study of lactic acid production with IC  
- high lactic acid production  
- high biomass/cell volumetric productivity | Norton et al., 1994 |
| 1-stage continuous/ κ-carrageenan, LSB/ milk | *Lc. lactis, Lc. diacetylactis, Lc. lactis; Leuconostoc mesenteroides* | Continuous fermentation for fresh cheese production  
- feasible system for constant milk pre-fermentation in fresh cheese manufacture | Sodini et al., 1997 |
| 1-stage continuous/ alginate/ MRS (no acetate, lactose) | *Lc. diacetylactis* | Comparison of chemostat IC vs free cell batch  
- high cell productivity  
- change of redox state | Cachon et al., 1998 |
| 1-stage continuous/ κ-carrageenan, LSB/ whey permeate, YE | 2 strains *Lc. lactis* and 1 *Lc. diacetylactis* | Production of mixed starter culture  
- long-term biological stability for mixed starter culture | Lamboley et al., 1999 |
| Repeated batches/ chitosan treated polypropylene/ medium of glucose, YE minerals | *Lb. plantarum* | Physiological analysis of IC  
- shift from homo- to heterofermentative metabolism  
- changes in cell morphology | Krishnan et al., 2001 |
| Repeated batches/ κ-carrageenan, LSB/ MRSC + CaCl₂ | *B. longum; Le diacetylactis* | Study effect of IC on co-cultivation  
- successful co-cultivation of mixed culture containing a non-competitive *B. longum* strain | Doleyres et al., 2002a |
<table>
<thead>
<tr>
<th>1-stage continuous/ gellan/ MRS and cysteine (MRSC), whey permeate (WP)</th>
<th>B. longum</th>
<th>Comparison of chemostat IC vs free cell batch growth - higher volumetric productivity</th>
<th>Doleyres et al., 2002b</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-stage continuous κ-carrageenan, LSB/ MRSC, lactose, CaCl$_2$</td>
<td>B. longum; Lc. diacetylactis</td>
<td>Effect of co-culture in chemostat IC on cell physiology - increased stress tolerance to freeze drying, H$_2$O$_2$ and gastrointestinal conditions</td>
<td>Doleyres et al., 2004a</td>
</tr>
<tr>
<td>2-stage continuous silicone rubber solid carriers/ whey permeate, YE</td>
<td>Lb. rhamnosus</td>
<td>Biomass production chemostat IC vs free cells - induction of cell aggregation</td>
<td>Bergmaier et al., 2005</td>
</tr>
<tr>
<td>2-stage continuous κ-carrageenan, LSB/ whey permeate, YE</td>
<td>Lb. helveticus</td>
<td>Modelling lactic acid production with chemostat IC - high lactic acid production - physiological adaptation</td>
<td>Schepers et al., 2006</td>
</tr>
<tr>
<td>1-stage continuous/ κ-carrageenan, LSB/ milk</td>
<td>Lb. rhamnosus; Lc. diacetylactis; P. acidilactici; Lc. cremoris</td>
<td>Test effect of mixed cultures with antagonistic properties in chemostat IC - enhanced acidification and tolerance to nisin</td>
<td>Grattepanche et al., 2007</td>
</tr>
<tr>
<td>2-stage continuous/ gellan, xanthan/ MRS</td>
<td>Lb. delbrueckii</td>
<td>Study the effect of chemostat IC and culture time - changes in cell morphology/aggregation - enhanced survival to freeze drying</td>
<td>Koch, 2006</td>
</tr>
<tr>
<td>1-stage continuous/ gellan, xanthan/ MRS + H$_2$O$_2$</td>
<td>B. longum</td>
<td>System to apply selective pressure to continuous IC - selection of H$_2$O-resistant mutant strain(s)</td>
<td>Mozzetti, 2009</td>
</tr>
</tbody>
</table>
1.7.2 Stabilization of probiotics

1.7.2.1 Microencapsulation

Microencapsulation is a technology for packing solids, liquids or gaseous material in small sealed capsules, which can release their contents at controlled rates under specific conditions (Shahidi and Han, 1993). The principle of microencapsulation has initiated numerous efforts in the last two decades to embed sensitive metabolically active probiotic strains in microcapsules or microparticles in order to increase stability during shelf-life and consumption. Encapsulation was shown to protect probiotic bacteria from oxygen, acidic environments in food products, refrigerated storage, and simulated gastro-intestinal conditions (Doleyres and Lacroix, 2005; Anal and Singh, 2007).

Different techniques and materials have been used for microencapsulation of probiotic cultures. Cells encapsulated in gel beads are achieved by entrapping probiotic cells within a polymeric matrix such as pectin, gellan gum, κ-carrageenan-locust bean gum and alginate. Gel capsules containing probiotics can be produced by various methodologies such as emulsion or extrusion techniques (Champagne and Fustier, 2007; Talwalkar and Kailasapathy, 2003). Several studies reported protective effects of microencapsulation and high survival of sensitive bifidobacteria and lactobacilli in plant polymer matrices (KrasaeKoOpt et al., 2003), particularly against oxygen (Talwakar and Kailasapathy, 2003), acidic environment (Sun and Griffiths, 2000), freezing (Shah and Ravula, 2000), refrigerated storage of ice cream and milk (Hansen et al., 2002) and yoghurt (Adhikari et al., 2003; Adhikari et al., 2000; Sultana et al., 2000) and during simulated gastro-intestinal tests (Lee and Heo 2000; Ding and Shah, 2007; 2009). Although promising on a laboratory scale, the technologies developed to produce gel beads present serious difficulties for large-scale production such as low production capacity and large bead diameters (2-5 mm), that
can affect food texture (Hansen et al., 2002). Moreover, the addition of these polysaccharides is not permitted in yogurts or fermented milks in some European countries (Picot and Lacroix, 2004). Beside the limitations in up-scaling and interference with textural and sensorial properties of food products (Anal and Singh, 2007), the proper release of viable cells at the target site is also an obstacle in the probiotic approach. In their recent review, de Vrese and Schrezenmeir (2008) reported that alginate capsules containing probiotics did not release their content after administration to human and pigs and considered this type of encapsulation although frequently used, as unsuitable as carrier for probiotic bacteria.

Another technique to encapsulate probiotics is the spray-coating technology for large scale production, which is widespread in industries for diverse commercial products (Anal and Singh, 2007; Champagne and Fustier, 2007). But since the technology is difficult to master and most information on the technology is proprietary to industry, there is only limited access for academic research (Champagne and Fustier, 2007). Spray coating consists of suspending, or fluidizing, particles of a core material in an upward stream of air and applying an atomized coating material (lipid-based, proteins or carbohydrates) to the fluidized particles (Augustin and Sanguansri, 2008). One particular patent claims improved stability of spray-coated cells to simulating gastric conditions, high temperature and during storage in powdered milk compared to uncoated freeze dried cells. In addition the coated cells had better resistance to compression (US patent 2003/0109025).

### 1.7.2.2 Spray drying

Dairy starter and probiotic cultures are mainly preserved and distributed in frozen or dried form. Frozen cultures occupy large volume and require storage at sub-zero temperatures causing high
costs of storage, shipping and energy and hence preservation in dried form is sometimes preferred (Johnson and Etzel, 1995). Transformation of bacteria to a dried form through the physical removal of water is a critical step since bacteria require a water activity ($a_w$) of about 0.98 in the product matrix for survival and growth. Preservation of a viable state requires either high $a_w$ for metabolic activity or low $a_w$ to survive in dormant state in powders (Paul et al., 1993). Drying of bacterial suspension is mainly assessed by freeze- or spray drying.

Freeze drying is widely used for formulation of starter and probiotic cultures but it is an expensive process with low yields, and as such spray drying offers an alternative inexpensive approach yielding higher production rates (Zamora et al., 2006). Spray drying is energy-efficient and an established tool in food industries for the production of milk powders and instant coffee. Despite restrictive process conditions for microorganisms (inlet reaching $\geq 180 ^\circ C$), the rapidity of drying combined with the possibility to dry large amounts of bacterial cultures has caught the attention of research and industry in recent years (Zamora et al., 2006; Chàvez and Ledeboer, 2007; Meng et al., 2008).

### 1.7.2.2.1 Principle of spray drying

During spray drying, feed solution is transformed from a fluid state into a dried form by spraying the feed into a hot drying medium (Figure 4). The spray drying process can be divided into two phases. First, the constant drying rate period just after the feed solution is dispersed by a fluid nozzle or atomizer and encounters the hot air in the drying chamber. The interior of the droplets is not challenged to heat exposure due to the cooling effect of evaporation. During the subsequent falling drying period, the temperature of the particles increases but does not reach values of the inlet temperature, because the remaining moisture content compensates exposure to heat until the
entire water is evaporated. Hence, the residence time of the droplets in the drying chamber drives the moisture content of the particles, as well as the survival of the microorganisms within the particles (Santivarangkna et al., 2007). It is generally accepted that bacteria in the dried particle are exposed to no higher temperatures than the outlet temperature, which in turn constitutes the crucial factor for bacterial survival to spray drying (Gardiner et al., 2000; Lian et al., 2002). The construction of the spray cylinder is designed to allow adequate residence time and droplet trajectory distance for achieving the heat and mass transfer. After the drying of the spray in the chamber, the majority of the dried product falls to the bottom of the chamber. The dried product can either be discharged continuously from the bottom of the spray cylinder or passed into a solid-gas separator (cyclone) where the solids are recovered from the gas stream. The powder is then collected at the bottom of the glass container. Very fine particles which do not precipitate in the glass container are usually collected in an outlet filter connected in series to the cyclone. The operating conditions and dryer design are selected according to the drying characteristics of the product and powder specification. The main parameters for powders are the residual moisture content and the particle size. Moisture content depends mainly on evaporation rate and the dryer $\Delta T$ (inlet air temperature minus the outlet air temperature), which in turn dictates the amount of drying air needed and ultimately the sizing and cost of almost all of the system components. The particle size requirement affects the choice of atomization method and can also affect the size of the dryer.

The system allows many heat sensitive products (enzymes, microorganisms, volatile aroma compounds, etc.) to be spray dried and transformed to powders with low moisture content.
Figure 4  Pathways of air and feed during spray drying

(Left) Path of the drying air in the spray dryer Büchi B290. Cold air is aspirated through the air inlet tunnel (1) and then electrically heated (2) prior to the atomization of the feed solution by the two-fluid nozzle (3). Dispersed feed enters the spray cylinder (4), in which drying of the droplets into solid particles takes place. Dried powder is separated from fine particles in the cyclone (5) and collected in the glass container (6). The outlet filter (7) is placed in series with the cyclone to remove fine particles and to prevent them from entering aspirator (8) which generates the air flow. Temperatures are measured in the entrance of spray cylinder (9, inlet temperature) and in the intermediate piece between spray cylinder and cyclone (10, outlet temperature).

(Right) Path of the feed solution and pressurized air in the spray dryer in co-current mode. Feed solution (A) conveyed by peristaltic feed pump (B) and atomizing air (D, inlet) are passed separately to the nozzle head (C), where the atomization of the feed solution into fine droplets takes place. The co-current two-fluid nozzle is located at the centre of the upper part of the spray cylinder. Atomization is created by compressed hot air at a pressure of 0.5 to 2 bar. Powders produced with this adjustment have particle size ranged from 5 to 15 µm on average.
1.7.2.2 Parameters affecting cell viability during spray drying

The drying process causes cell damage and decreased viability due to mechanical, heat, osmotic stress, exposure to oxygen, and the removal of bound water (Meng et al., 2008; To and Etzel, 1997). The decrease of water availability inside or in the vicinity of the dried cells affects the physiology of bacteria. Removal of water eventually results in slow down of bacterial metabolism or stops it entirely after which bacteria enter a dormant state. Possible damages caused by the dehydration are:

- increased permeability of cell wall/ cytoplasmic membrane to extracellular or leakage of cytoplasmic compounds due to lesions in cell envelope (Texeira et al., 1996).
- transformation of liquid crystalline structure of phospholipid bilayer to gel phase and incomplete reverse rehydration may cause leaking and disturbance of molecule transport systems (Oliver et al., 1997).
- peroxidation of lipid cause membrane leakage causing loss of plasmids or increased influx of DNase into cells affecting DNA integrity (Meng et al., 2008).
- conformational changes of enzymes may block or modify active binding sites resulting in inactivation of the enzyme (Prestrelski et al., 1993; Burin et al., 2004).
- changes in the lipid bilayer of cell membrane causing displacement, denaturation or loss of membrane-bound proteins (Texeira et al., 1996).

Poor survival of LAB and bifidobacteria during drying are substantial draw-backs for this preservation method and strategies to optimize cell viability are thus required (Meng et al., 2008; Santivarangkna et al., 2007). Survival of heat sensitive LAB and bifidobacteria during spray drying depends on several factors such as the drying carrier, bacterial growth phase, the particular
probiotic strain used and mainly on the outlet temperature of the drier. Adjustment and optimization of these parameters affects bacterial survival to spray drying as shown in Table 3. Stabilizing compounds for drying and shelf live are hydrophilic polyhydroxy substrates like sugars which partly substitute missing water molecules in membranes and proteins (Leslie et al., 1995, Oliver et al., 1997, Corcoran et al., 2004). Reconstituted skim milk (RSM) as a carrier contains lactose as a possible water substituent and was shown to be suitable for drying of probiotics (Corcoran et al., 2004; Ananta et al., 2005). Besides lactose, skim milk proteins in parallel with calcium may prevent cellular injury by stabilizing phospholipid bilayers and shielding the membrane-bound proteins by formation of a protective coating (King and Su, 1993; Castro et al., 1995).

In recent years, numerous studies on the optimization of spray drying of probiotics have been performed to either increase cell robustness or to modify the drying parameters to enhance the yields of viable cells in spray dried powders (Table 3).
Table 3  
Survival rates of lactobacilli and bifidobacteria during spray drying

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Survival (%)( ^a )</th>
<th>(T_{\text{out}}(T_{\text{out}}))</th>
<th>(T_{\text{in}}(T_{\text{in}}))</th>
<th>Feed solution</th>
<th>Reference</th>
<th>Special remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Lb.\ acidophilus)</td>
<td></td>
<td>75, 80, 85 °C</td>
<td>75, 80, 85</td>
<td>25 % solid milk</td>
<td>Espina and Packard, 1979</td>
<td>Moisture content of powder (M.C.) 3.5 - 6.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSM (25%)</td>
<td>8.2, 2.4, 0.5</td>
<td>40 % solid milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSM (40%)</td>
<td>3.7, 0.5, 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S.\ salivarius) ssp. (\text{thermophilus, Lb. delbrueckii ssp. bulgaricus})</td>
<td>2 - 0.1</td>
<td>60, 70, 80</td>
<td>140 – 180</td>
<td>yoghurt, 13.63% solid non fat</td>
<td>Kim and Bhownik, 1990</td>
<td></td>
</tr>
<tr>
<td>(Lb.\ helveticus)</td>
<td>15</td>
<td>82, 120</td>
<td>220</td>
<td>19% maltodextrin</td>
<td>Johnson and Etzel, 1995</td>
<td>(T_{\text{out}}) adjustment using feed flow rate</td>
</tr>
<tr>
<td>(Lb.\ bulgaricus)</td>
<td>n. i.</td>
<td>62-105</td>
<td>200</td>
<td>skim milk</td>
<td>Texeira \textit{et al.}, 1995</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n. i.</td>
<td>80</td>
<td>200</td>
<td>40% maltodextrin in water or RSM 11%</td>
<td>Texeira \textit{et al.}, 1995</td>
<td></td>
</tr>
<tr>
<td>(Lb.\ bulgaricus)</td>
<td>n. i.</td>
<td>70</td>
<td>200</td>
<td>RSM</td>
<td>Texeira \textit{et al.}, 1996</td>
<td></td>
</tr>
<tr>
<td>(Lc.\ lactis, Lb. casei, S.\ thermophilus)</td>
<td>3.0 – 0.4, 14.7 – 13.0, 34 – 5.0</td>
<td>70 – 90</td>
<td>220</td>
<td>25% (w/w) solid maltodextrin</td>
<td>To and Etzel, 1997</td>
<td></td>
</tr>
<tr>
<td>(Lb.\ lactis)</td>
<td>14.5 – 0.6</td>
<td>68</td>
<td>160 – 200</td>
<td>20% RSM</td>
<td>Mauriello \textit{et al.}, 1999</td>
<td>Flow rate feed: 10, 13, 17 ml/min</td>
</tr>
<tr>
<td>(S.\ thermophilus, Lb.\ delbrueckii)</td>
<td>54.7 – 51.6, 15.8 – 13.7</td>
<td>60 – 80</td>
<td>180</td>
<td>yoghurt</td>
<td>Bielecka and Majkowska, 2000</td>
<td>Water evaporation capacity 1645 kg/h</td>
</tr>
<tr>
<td>(Lb.\ paracasei) (Lb.\ salivarius)</td>
<td>65.0, 1.0</td>
<td>60-120</td>
<td>170</td>
<td>20% RSM</td>
<td>Gardiner \textit{et al.}, 2000</td>
<td>M.C. ca. 4%</td>
</tr>
<tr>
<td>(Lb.\ paracasei)</td>
<td></td>
<td>Heat: Control 4, Adapted 23</td>
<td>85 – 105</td>
<td>20% RSM</td>
<td>Desmond \textit{et al.}, 2001</td>
<td>M.C. 1.7 - 3.3 % (T_{\text{out}}) adjusted with feed flow rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salt: Control 7, Adapted 33</td>
<td>170</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Bifidobacteria)</td>
<td>16.6</td>
<td>45</td>
<td>100</td>
<td>10% (encapsulation)</td>
<td>O’Riordan \textit{et al.}, 2001</td>
<td></td>
</tr>
<tr>
<td>(B.\ infantis) (2 strains), (B. longum) (3 strains)</td>
<td>82.6 – 0.1</td>
<td>50-60</td>
<td>100</td>
<td>Gelatin, gum arabic, skim milk, soluble starch, 10% RSM</td>
<td>Lian \textit{et al.}, 2002</td>
<td>M.C. 7-9% Carrier dependent survival:(c): RSM&gt; Gelatin&gt; gum&gt; starch</td>
</tr>
</tbody>
</table>
Table continued

<table>
<thead>
<tr>
<th><strong>Lb. salivarius, Lb. sakei</strong></th>
<th>100</th>
<th>1000</th>
<th>105</th>
<th>170°C</th>
<th>20% RSM or 10% RSM with 10% gum accacia</th>
<th>Silva et al., 2002</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lb. paracasei NFBC338</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>T&lt;sub&gt;out&lt;/sub&gt; adjusted with feed flow rate</strong></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Desmond et al., 2002</td>
</tr>
<tr>
<td><strong>Lb. acidophilus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>T&lt;sub&gt;in&lt;/sub&gt; 130 °C, T&lt;sub&gt;out&lt;/sub&gt; 75 °C</strong></td>
<td></td>
</tr>
<tr>
<td><strong>B. lactis Bb12</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Favaro-Trindale and Grosso, 2002</td>
</tr>
<tr>
<td><strong>Lb. rhamnosus GG, Lb. rhamnosus E800, Lb. salivarius</strong></td>
<td>50, 50</td>
<td>50, 50</td>
<td>0.7, 0.7</td>
<td>85-90</td>
<td>170</td>
<td>RSM 20% or 10% RSM with 10% polydextrose</td>
</tr>
<tr>
<td><strong>B. brevis</strong></td>
<td>25.7</td>
<td>25.7</td>
<td>80</td>
<td>160</td>
<td>10 % Whey protein</td>
<td>Picot and Lacroix, 2004</td>
</tr>
<tr>
<td><strong>B. longum</strong></td>
<td>1.4</td>
<td>1.4</td>
<td>70</td>
<td>200</td>
<td>11% RSM</td>
<td>Silva et al., 2004</td>
</tr>
<tr>
<td><strong>St. thermophilus</strong></td>
<td></td>
<td>60 – 90</td>
<td>100</td>
<td></td>
<td>Fermented soy milk</td>
<td>Wang et al., 2004</td>
</tr>
<tr>
<td><strong>Lb. acidophilus</strong></td>
<td></td>
<td>75 °C</td>
<td></td>
<td></td>
<td><strong>M.C. 2.5 - 3.2%</strong></td>
<td>Study about influence of mixed cultures on survival to spray drying</td>
</tr>
<tr>
<td><strong>B. infantis, B. longum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lb. delbrueckii ssp. bulgaricus</strong></td>
<td></td>
<td>70</td>
<td>200</td>
<td>11% RSM</td>
<td>Silva et al., 2004</td>
<td></td>
</tr>
<tr>
<td><strong>St. thermophilus</strong></td>
<td></td>
<td>60 – 90</td>
<td>100</td>
<td></td>
<td>Fermented soy milk</td>
<td>Wang et al., 2004</td>
</tr>
<tr>
<td><strong>Lb. rhamnosus GG</strong></td>
<td></td>
<td>60 – 90</td>
<td>100</td>
<td></td>
<td>RSM 20% modified with oligofructose and polydextrose</td>
<td>Ananta et al., 2005</td>
</tr>
<tr>
<td><strong>B. breve</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>M.C. 2.7 – 4.5 %</strong></td>
<td>T&lt;sub&gt;out&lt;/sub&gt; adjusted by changing T&lt;sub&gt;in&lt;/sub&gt;.</td>
</tr>
</tbody>
</table>
Table continued

<table>
<thead>
<tr>
<th></th>
<th>Survival Rate</th>
<th>T&lt;sub&gt;in&lt;/sub&gt;</th>
<th>T&lt;sub&gt;out&lt;/sub&gt;</th>
<th>Carrier</th>
<th>Source</th>
<th>M.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bifidobacterium</em> subsp.</td>
<td><strong>12 – 102</strong></td>
<td>85 – 90</td>
<td>170</td>
<td>RSM 20% (w/v) or RSM (10%, w/v) and gum acacia (10%, w/v)</td>
<td>Simpson <em>et al.</em>, 2005</td>
<td><strong>2.7 – 4.2 %</strong></td>
</tr>
<tr>
<td>Lb. salivarius</td>
<td>BetL&lt;sup&gt;a&lt;/sup&gt; <strong>1.4</strong></td>
<td>80 – 85</td>
<td>170</td>
<td>RSM</td>
<td>Sheehan <em>et al.</em>, 2006</td>
<td>“BetL” plasmid containing listerial betaine uptake system BetL&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>BetL&lt;sup&gt;c&lt;/sup&gt; 0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lb. acidophilus</td>
<td><strong>1.5</strong></td>
<td>58 – 79</td>
<td>100 – 120</td>
<td>RSM, whey permeate with guarana gum</td>
<td>Riveros <em>et al.</em>, 2009</td>
<td><strong>6.3 %</strong></td>
</tr>
<tr>
<td>Lb. rhamnosus GG;</td>
<td>MSG., RSM</td>
<td>60 – 75</td>
<td>varying</td>
<td>Trehalose (20%, w/v) supplemented with 12.5 g/l monosodium glutamate (MSG)</td>
<td>Sunny-Roberts and Knorr, 2009</td>
<td><strong>3.6 – 4.4 %</strong></td>
</tr>
<tr>
<td>Lb. rhamnosus E-97800</td>
<td><strong>69, 75</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>23, 55</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup> Different survival rates correspond to different spray drying parameters (e.g. strain, carrier, T<sub>out</sub> etc.)

<sup>b</sup> Proximate values estimated from data in figures.

<sup>c</sup> Series of descending survival for each carrier for the majority of tested strains

<sup>d</sup> Genetically modified organism

n.i.: not indicated
1.7.2.2.3 Parameters affecting storage of dried powders

During powder storage, microbial stability is affected by parameters like temperature, oxygen content, light exposure and storage materials. Moreover, powder characteristics such as moisture content, powder composition and relative humidity can influence the glassy state of the carrier medium and eventually the viability of microorganisms during shelf life. Glass transition temperature \( T_g \) is the critical temperature at which a glass-forming liquid transforms from a rubbery to a glassy state. The specific \( T_g \) of sugar containing powders plays an important role in the stability of powders containing microorganisms and is dependent on storage temperature and moisture content of the powder (Figure 5). High viscosity of sugar glasses below or around their glass transition temperature retards molecular mobility and reactivity resulting in a stabilizing effect of biological systems (Buitink et al., 2000). Consequently, the storage of dried cultures at temperatures below the glass transition temperatures of sugars in powder mixtures enhance shelf-life stability (Vega and Roos, 2006; Santivarangkna et al., 2008). High storage temperature and remaining water in dried powders have thus a detrimental effect on cell viability, as shown for dried lactobacilli and bifidobacteria in skim milk (Gardiner et al., 2000; Silva et al., 2002; Zayed and Roos, 2004; Simpson et al., 2005).

Cell damage during storage may be caused by oxidation of membrane lipids. The presence of allyl-groups in unsaturated fatty acids causes instable membrane properties during storage since double-bonds are targets to oxidation and lead to formation of cytotoxic hydroperoxides (Texeira et al., 1996). Progressive changes of lipid composition were observed during storage of lactobacilli (Castro et al., 1996) and cells with damaged bacterial membranes became consequently more sensitive to NaCl (Texeira et al., 1996). The detrimental effects on cell viability during storage require treatments to stabilize and improve stability of spray dried probiotic powders to make spray drying a cost efficient alternative to traditional freeze drying.
Figure 5  A simplified state diagram shows the glass transition curve which relates the glass transition temperature and moisture content. Molecular mobility and deleterious reaction rates in the glassy state are extremely low, while they increase at the storage conditions above the curve (rubbery state) (Santivarangkna et al., 2008).

Studies on optimization of storage stability of bacteria in dried powders focused on supplementation or substitution of the carrier medium (usually RSM) by oligosaccharides or oxygen scavenger compounds. Although sugars are believed to be the major components affecting glass formation, it has also been shown that polypeptides can significantly alter the glass properties of sugars (Buitink et al., 2000) and therefore play the important role in glass formation. This could explain why skimmed milk powder containing a large number of milk proteins is considered as an efficient desiccation protectant (Hubalek, 2003). Powder stability of lactobacilli dried in RSM supplemented with prebiotic substances or polydextran did not improve
bacterial viability during storage compared to RSM alone (Corcoran et al., 2004; Ananta et al., 2005). Addition of anti-oxidants such as ascorbic acid and monosodium glutamate to powders of *Lb. delbrueckii* ssp. *bulgaricus* stored at different temperatures showed ambiguous results and even destabilizing effect at 20 °C (Texeira et al., 1995) concluding that protective agents during the dehydration process do not necessarily support viability of cells during storage (Crowe et al., 1987). Stability of probiotic powders can be improved by proper packaging. The use of aluminium-based packaging material that protects the powder from exposure to light, moisture and oxygen supports storage stability of dried probiotics (Nagawa et al., 1988; Ishibashi and Shimamura, 1993). Shelf-life stability of probiotic bacteria is strain dependent and not related to the survival during drying (Gardiner et al., 2000; Simpson et al., 2005; Sunny-Roberts and Knorr, 2009). Hence, a careful selection of robust strains should be performed with a view to obtained strains which have stable shelf life properties. Variation of stability during drying and storage among strains of the same species can impede the ability to extrapolate results across entire subspecies or species. Predictions are thus of limited significance. Consequently, studies on the effect on survival of changing parameters in drying and storage applications have to be performed strain by strain. This is laborious and time consuming, particularly during storage tests, and hence new technologies for efficient simultaneous screening methods are required to increase output of such experiments.

1.7.2.2.4 Rehydration

The rehydration of probiotic powders is the final critical step during stabilization of probiotic powders and revitalization conditions can have significant influence on the recovery of viable cells since bacterial membranes become more susceptible to environmental stresses (Texeira et al., 1996). Revitalization of cells and can be divided into four steps: wetting, submersion,
dispersion and dissolving (Freudig et al., 1999). Wetting of the particles is very often the reconstitution controlling step and the rate of recovery depends on various factors including osmolarity, pH, and nutritional energy as well as on temperature and volume (Carvalho et al., 2004, Vega and Roos, 2006). Slow rehydration under controlled conditions was shown to improve cell viability compared to immediate rehydration (Poirier et al., 1999). The medium to rehydrate cells also affects recovery rate. For instance, complex media such as 10% (w/v) RSM and PTM media consisting of peptone, tryptone and meat extract positively affected bacterial cell recovery compared to minimal media such as phosphate buffer, sodium glutamate and water (Costa et al., 2000). In other studies the use of the same cryopreservation and rehydration solution resulted in increased viability (Ray et al., 1971; Abadias et al., 2001). A possible explanation is that such a solution provides a high osmotic pressure environment which controls the rate of hydration, and thus avoids osmotic shock (Morgan et al., 2006).

1.7.3 Enumeration of viable probiotic cells

Apart from advanced production and downstream processing technologies, the criterion of minimal levels of viable cells within probiotic products requires also adequate quantification methods to detect the viable probiotic strains in functional food products. The application of rapid and reliable methods for enumeration of probiotics in mono- and mixed cultures is important to validate quality of products containing probiotics. Cell quantification is generally performed by plate counting on selective media to distinguish the microorganisms. However, this method has several limitations including cell clumping, inhibition by neighboring cells, and it is time consuming (Auty et al., 2001; Breeuwer and Abee, 2000; Bergmaier et al., 2005). Another aspect concerns the lack of a standard medium to distinguish bifidobacteria from other LAB and
expressed thus the need for alternate, non-culture based technologies for adequate cell enumeration (Talkalwar and Kailaspathy, 2003; Masco et al., 2005). The use of fluorescent stains alone or in combination with enzymatic assays enabled the differentiation between viable, metabolic active, damaged, dormant, viable but not cultivable, and dead bacterial cells and were used to quantify viable bacteria (Breeuwer and Abee, 2000; Alakomi et al., 2005). Molecular tools like fluorescence in situ hybridization (FISH) and flow cytometry were successfully applied to estimate viable cells in probiotic products (Maukonen et al., 2006). Moreover, flow cytometry can distinguish between subpopulations of stressed versus unstressed bifidobacteria (Ben Amor et al., 2002). However, culture-based and fluorescent methods demand single cells suspension because cell in clusters or chains lead to biased quantification of viable cell counts (Daley et al., 1977; Nebe-von-Caron et al., 2000; Bibiloni et al., 2001).

There exists however alternative methods to detect or quantify bifidobacteria in fecal samples and pharmaceutical or probiotic products. Technologies to detect bifidobacteria are based on DNA fingerprinting approaches such as denaturing gradient gel electrophoresis (DGGE), pulsed-field gel electrophoresis (PFGE). Quantitative approaches relate to the use of quantitative real time PCR (qRT-PCR) (Vitali et al., 2003; Gueimonde et al., 2004; Masco et al., 2005; Matsuda et al., 2007; Masco et al., 2007). But these methods are unable to provide information on the metabolic activity or viability of the microorganisms since DNA or rRNA are too stable to be used as viability markers (Josephson et al., 1993; Sheridan et al., 1998). mRNA on the other hand with short half-life times can be considered as accurate viable cell marker (Belasco et al., 1986; Hellyer et al., 1999). The quantification of mRNA using qRT-PCR has been recently applied in medical applications to enumerate viable pathogenic microorganisms (Birmingham et al., 2008, Coutard et al., 2005), but to date no such approach with mRNA has been applied to probiotic bacteria. Growing numbers of accessible genome sequences of food related microorganisms and
progressing microarray technology will enlarge the knowledge about gene expression profiles of bacteria in different environments which will raise the number adequate target mRNAs to accurately quantify viable bacteria in various environments.

1.8 Hypothesis and background of this study

1.8.1 Background and general objective

Bifidobacteria are fastidious bacteria and sensitive to various environmental stresses during production, downstream processing, incorporation in probiotic food products, storage and transit through upper GIT. There is a general demand by food industries for advanced technologies to enhance cell robustness and to improve selection procedure of candidate strains to meet minimal viable cell numbers in products of prospering probiotic food market. Furthermore, the limitations of accurate enumeration of viable cells require alternative methods to accurately quantify viable cells independent of their morphological state.

The aim of the first part of the dissertation was to produce and characterize stress tolerant \textit{B. longum} NCC2705 strains using IC during continuous culture (Chapter 2). Continuous IC culture induced formation of large cell aggregates which made physiological characterization with traditional plate count method very difficult. Hence, we developed a new method to enumerate viable \textit{B. longum} NCC2705 irrespective of the morphological state (Chapter 3). The method based on the quantification of the mRNA of a constitutively expressed housekeeping gene with real-time PCR technology. With the help of this method, we were able to characterize survival of aggregated cells produced with IC technology to lethal bile and heat stresses (Chapter 2).

In the last part of the doctoral thesis, we chose a different approach. Instead of producing stress tolerant \textit{B. longum} strains, we developed a high throughput screening method to identify \textit{B.}
strains with intrinsic resistance to stresses occurring during spray drying and storage (Chapter 4). Molecular biological tools allowed a differentiation of 22 *B. longum* strains of which we were able identify the best survivor strain after spray drying and storage. The method was then validated by comparing survival of the selected strain with a poor surviving strain.

### 1.8.2 Specific objectives

a. Characterization of *B. longum* NCC2705 produced with ICT in continuous culture:

- Perform a long-term fermentation with immobilized cells in a two-stage fermentation system for 20 days
- Characterize biomass production, cell metabolism, and membrane composition
- Analyze the effect of cell immobilization and continuous culture on cell physiology: tolerance to antibiotics, heat and porcine bile compared to free cell batch culture

b. Development of a real time PCR method to quantify viable *B. longum* NCC2705 cells in large aggregates:

- Generate calibration curves with level of transcript of two housekeeping genes as a function of cell concentration
- Validate target fragments of transcripts as viability marker
- Apply the method to quantify viable cells in large aggregates from continuous IC culture
c. Development of a fast screening method for selection of *Bifidobacterium longum* strains resistant to spray drying and storage in dry powder:

- Establish strain specific molecular profiles using randomly amplified polymorphic DNA (RAPD)
- Perform spray drying and storage experiments of mixtures of *B. longum* strains
- Validate the selection of resistant strain with single strain spray drying and storage experiment

### 1.8.3 Hypotheses

- Immobilization and continuous culture of *Bifidobacterium longum* NCC2705 improves volumetric productivity and tolerance to bile salt, heat and antibiotics.

- Quantification of transcript levels of a housekeeping gene with qRT-PCR allows accurate enumeration of viable *Bifidobacterium longum* NCC2705 cells of different morphologies.

- The simultaneous spray drying and storage of mixed *B. longum* strains allows the rapid identification of the most robust strains using RAPD PCR technology.
2 Improved tolerance to bile salts of aggregated

B. longum NCC2705 produced during continuous
culture with immobilized cells
2.1 Abstract

The effect of cell immobilization and continuous culture was studied on selected physiological
and technological characteristics of *Bifidobacterium longum* NCC2705. *B. longum* NCC2705 was
entrapped in gellan/xanthan gel beads and cultivated for 20 days with and without glucose
limitation in a two stage continuous fermentation system, with a first reactor (240ml) containing
immobilized cells, and a second reactor (1800ml) inoculated with free cells produced in the first
reactor. Continuous immobilized cell (IC) cultures with and without glucose limitation exhibited
formation of macroscopic cell aggregates after 12 and 9 days, respectively. Auto-aggregation
resulted in underestimation of viable cell counts by plate counts by more than 2 log units CFU/ml
compared to cell counts obtained with a cell-aggregate-independent method using real time PCR
technology. Cells in the effluent of continuous IC culture showed enhanced tolerance to porcine
bile salts and to aminoglycosidic antibiotics compared to stationary-phase cells produced during
free-cell batch fermentations. However, tolerance to lethal heat treatment was not different than
that from batch control culture. Changes in the cell membrane composition of continuously
produced cells such as the decrease of the ratio of unsaturated divided by saturated fatty acid
content of 0.57 ± 0.11 were observed compared to 1.74 ± 0.00 of membranes from batch control
culture.

Cells produced during continuous immobilized-cell cultures exhibited changes in cell membranes
which may induce formation of cell aggregates and tolerance to bile salts and aminoglycosidic
antibiotics. Cell immobilization in combination with continuous culture can be used to produce
probiotic bacteria with improved stress tolerance.
2.2 Introduction

Probiotics are “living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition” (WHO/FAO 2001). Bifidobacteria are widely used as probiotics in functional food products (Leahy et al., 2005). Bifidobacteria are sensitive to various factors during production and downstream processing and need to adapt to competitive and changing environment in the human gastrointestinal tract to maintain viability (Lacroix and Yildirim, 2007). Apart from safety issues, selection of potential probiotic candidate strains relies on technological as well as functional properties. Among others, resistance to oxygen, heat and gastro-intestinal conditions during production and consumption as well as the ability to adhere to intestinal mucosa are thought to be important to provide beneficial health effects for the host (Ouwehand et al., 2002; Salminen et al., 1999).

Different strategies such as pre-treatment of bacterial cells with sub-lethal stresses (Desmond et al., 2002; Maus and Ingham, 2003) or physical protection using whole cell encapsulation (Champagne et al., 2007) have been proposed to enhance stability of probiotics to stressful conditions. Doleyres et al. (2004a) reported for continuous co-culture of immobilized B. longum and L. lactis strains that cells produced in the effluent medium of the immobilized cell reactor exhibited significantly increased tolerance to hydrogen peroxide, freeze-drying, and simulated gastrointestinal conditions compared to free cells cultured in batch mode. Enhanced physiological properties upon cell immobilization were also reported for lactobacilli where tolerance to nisin Z and higher acidification activity were observed for continuously produced cells in mixed culture (Grattepanche et al., 2007). The enhanced stress tolerance of immobilized cells were tentatively attributed to changes in the microbial cell membranes of LAB and/or to non-specific stress adaptations due to steep gradients of inhibitory products, pH and biomass within gel beads.
(Masson et al., 1994; Trauth et al., 2001; Lacroix et al., 2005; Schepers et al., 2006). However, the exact mechanism conferring increased stress tolerance remains unclear.

In this work, we studied the production of *B. longum* NCC2705 and time stability of continuous cultures with immobilized cells (IC) in a two-stage fermentation system similar to that used by Doleyres et al. (2004a) for mixed cultures with a first small reactor containing cells immobilized on gel beads and a second large reactor connected in series and continuously inoculated from cells produced in the first reactor. *B. longum* NCC2705 was chosen as model strain because its genome has been sequenced (Schell et al., 2002). Furthermore, the probiotic characteristics, technological properties, and stress tolerance and adaptation of this strain have been thoroughly analyzed in the review of Klijn et al. (2005). The effect of cell immobilization and fermentation time on cell production, metabolism, and selected physiological characteristics (morphology, tolerance to bile salt, heat and antibiotics and membrane fatty acid composition) in the effluent broth of continuous IC cultures were measured and data were compared to that of stationary-phase cells produced from traditional free cell batch fermentations. Growth medium not supplemented with glucose induced starving conditions in both reactors and thus a second continuous fermentation was carried out in growth medium supplemented with extra-glucose.
2.3 Material and Methods

2.3.1 Strain and medium

*Bifidobacterium longum* NCC2705 was provided by the Nestlé Culture Collection (NCC; Nestlé Research Centre, Lausanne, Switzerland). Cells were grown under anaerobic conditions using atmosphere generation system packs (AnaeroGen, Oxoid, Pratteln, Switzerland) at 37 °C in de Man, Rogosa and Sharp medium (MRS; de Man *et al*., 1960) broth (BioLife, Milan, Italy) supplemented with 0.5 % (w/v) L-cysteine (Sigma-Aldrich, Buchs, Switzerland) (MRSC) and inoculated at 1 % (v/v) with an overnight pre-culture before use.

2.3.2 Cell immobilization

Gel beads were produced in a two phase dispersion process using gellan and xanthan gum according to Cinquin *et al*. (2004). Briefly, a polysaccharide mix was prepared containing gelrite gellan (2.5 % (w/v), xanthan (0.25 % (w/v)) powders and sodium citrate (0.2% (w/v)) (Sigma-Aldrich) resuspended in 500 ml pre-heated distilled water (90 °C) and mixed in a blender until dissolution. The polymer solution was then autoclaved for 15 min at 121 °C just prior to use for immobilization. The autoclaved polymer solution was cooled to 43 °C and inoculated aseptically with *B. longum* NCC2705 pre-culture (2%; v/v). Gel beads were formed using a 2 phase dispersion process by pouring the polymer solution into sterile hydrophobic phase (commercial sunflower oil) at 43 °C. Beads were hardened by cooling the suspension to 25 °C and by replacing the hydrophobic phase with 0.1 M solution of CaCl$_2$ (Sigma-Aldrich). Beads with diameters in the 1.0–2.0 mm range were selected by wet sieving and used for fermentation. The entire process was completed in aseptic conditions within 1 h.
2.3.3 Continuous fermentations

Two IC continuous cultures were performed for 20 days. Before starting the continuous fermentation, a bead colonization step was carried out in batch mode in a 500 ml bioreactor (Sixfors, Infors-HT, Bottmingen, Switzerland) containing 370 ml MRSC and 80 ml of inoculated gel beads. The colonization step was carried out at 37 °C for 16 h with CO$_2$ flushed in the headspace to ensure anaerobic conditions and agitation rate at 150 rpm by an inclined flat blade impeller. pH was maintained at 6.0 by addition of 5 M NaOH. Colonized gel beads were then aseptically wet sieved and transferred to a 500 ml bioreactor (R1) (Sixfors) containing 160 ml medium for continuous fermentation for a total volume of 360 ml. Flow rate of fresh medium was set at 360 ml/h using peristaltic pumps (Ismatec Reglo, Ismatec, Glattbrugg, Switzerland) during the 20 days of culture, corresponding to a dilution rate of 2.25 h$^{-1}$. Stirring was set at 200 rpm. A second reactor (R2) (Bioengineering, Wald, Switzerland) with a working volume of 1800 ml (dilution rate 0.2 h$^{-1}$) was operated in series with R1 and continuously inoculated by fermented broth from R1 (Figure 6). Stirring with two rushton type impellers in R2 was set at 250 rpm. The reactor volumes were chosen to yield a shorter residence time in R1 than in R2 (ca. 27 min and 5 h, respectively) for a high cell productivity in R1 containing a high immobilized cell concentration and operated at high dilution rate and final growth in R2 with longer residence time (Doleyres et al., 2004b). The pH in both reactors was maintained at 6.0 by addition of 5 M NaOH. Temperature was set at 37 °C and CO$_2$ was flushed in the headspace of both reactors to provide anaerobic conditions.

Fermented broth samples were taken daily from the two reactors for microscopy, dry biomass, HPLC analyzes, and viable cell enumeration by plate counts. Samples for stress tolerance assays were taken every 2 days, centrifuged (6,000 × g for 15 min at 4 °C), and pellets were resuspended.
in an equal volume of MRSC containing 10 % (w/v) glycerol (Sigma-Aldrich) prior to freezing in liquid nitrogen and storage at -80 °C. This step was required to test samples in blocks and correct for assay variability. HPLC samples were prepared by centrifuging 2 ml samples (10,000 × g for 1 min) and 1.5 ml of the supernatant was stored at -20 °C until analysis.

The first culture (ICC1) was run with unsupplemented MRSC leading to complete sugar consumption in R1 and R2 whereas the second culture (ICC2) was operated with MRSC supplemented with glucose (final concentration of 60 g/l) and anhydrous CaCl$_2$ at 1.5 g/l (Sigma-Aldrich). CaCl$_2$ was added to ensure gel bead-stability throughout the entire fermentation time because gel beads appeared soaked and consistency was weak at the end of ICC1. After sterilization, initial glucose concentration measured in fresh medium for ICC1 and ICC2 was 13.6 and 52.8 g/l, respectively.

![Diagram of the two-stage reactor setup for continuous fermentation with immobilized cells including tubings for feed inlet, reactor connection, medium outlet, emergency out, sampling ports, NaOH addition (pH: 6.0) and CO$_2$ injection for anaerobic conditions.](image)

**Figure 6** Diagram of the two-stage reactor setup for continuous fermentation with immobilized cells including tubings for feed inlet, reactor connection, medium outlet, emergency out, sampling ports, NaOH addition (pH: 6.0) and CO$_2$ injection for anaerobic conditions.
Chapter 2 - Immobilized cells

2.3.4 Batch fermentation

Batch fermentation was carried out in a glass bioreactor (Bioengineering) with a working volume of 2000 ml similar to R2 of the continuous fermentation. Unsupplemented MRSC was inoculated at 2 % (v/v) with twice sub-cultured *B. longum* NCC2705 and incubated for 16 h at 37 °C under anaerobic conditions by flushing CO₂ in the headspace. Mixing was set at 250 rpm and pH controlled at 6.0 by addition of 5 M NaOH. Growth was monitored by OD₆₀₀ and growth phases were set as follows: end exponential (9.5 h, OD₆₀₀ 4.3 ± 0.1), early stationary (12.5 h, OD₆₀₀ of 5.7 ± 0.4) and mid stationary growth (16 h, OD₆₀₀ 5.6 ± 0.4). Samples were collected and proceeded as described above. Batch fermentations were carried out in triplicate.

2.3.5 Viable cells enumeration

Viable cells in the fermented broth were enumerated by serially diluting fresh effluent of both reactors in 12 mM phosphate buffer saline with 0.05 % cysteine (w/v) (PBSC). Appropriate dilutions were drop plated in duplicate on MRS agar (1.5 %) (Becton Dickinson, Basel, Switzerland) supplemented with 0.05 % (w/v) cysteine. Plates were incubated anaerobically using atmosphere generation system packs (AnaeroGen, Oxoid, Pratteln, Switzerland) at 37 °C for 48 h.

Viable cell enumeration using qRT-PCR method was only performed for samples from R2 of ICC2 and bile- and heat tests of the same samples by quantifying levels of a 400 bp mRNA fragment of a housekeeping gene, *purB*, (see Chapter 3.3). This method was used to estimate viable cell counts in aggregated cell samples. Briefly, 0.5 ml of washed cell samples were put in 2-mL screw-cap tubes containing a mix of glass beads, phenol/chloroform 1:1 (v/v), sodium dodecyl sulfate 10 % (w/v) and 3 M sodium acetate (pH 5.2) (Sigma-Aldrich) and immediately
frozen in liquid nitrogen. Cells were disrupted by bead-beating in a homogenizer (FastPrep FP 120, Q-Biogene, Carlsbad CA, USA), four times for 40 sec at speed 4.0 intercalated with cooling periods on ice. Samples were then centrifuged to remove cell debris and glass beads. The aqueous phase containing RNA was transferred to a new tube and supplemented with a known amount of exogenous luciferase mRNA (Promega, Dübendorf, Switzerland) as a reference. Traces of phenol were removed by extraction with pre-chilled chloroform. The resulting aqueous phase was mixed with an equal volume of 70%-ethanol and applied to RNA purification column using RNeasy Mini Kit (Qiagen, Basel, Switzerland). Further purification was performed according to the manufacturer’s protocol including on-column DNase I digestion for 30 min followed by elution in 100 μl H2O. Purified RNA (max 100 ng) was applied to reverse transcription using the high capacity cDNA reverse transcription commercial kit (Applied Biosystems, Rotkreuz, Switzerland). cDNA was then applied to quantitative PCR using an ABI PRISM 7700 equipment with SYBR® Green PCR Master Mix (Applied Biosystems). The normalized levels of the 400 bp amplicon were then measured to determine viable cell concentrations along with prepared calibration curves.

2.3.6 Dry biomass determination

Dry biomass was determined according to Bergmaier et al. 2005 with slight modifications. 6 ml of thawed sample were centrifuged at 10,000 × g for 15 min at 4 °C. The pellet was washed twice with distilled water and transferred to a pre-weighed aluminum dish. The cells were dried for 20 h in an oven at 99 °C and weighed after cooling to room temperature (25 °C) in an exsiccator (Duran Group GmbH, Mainz, Germany). Values are single determinations.
2.3.7 Microscopic observation

Pictures of fresh cells from the fermentation were obtained using an optical microscopy at 1000-fold magnification (Leica DM, Leica Microsystems, Wetzlar, Germany) by an attached camera (Leica DFC280) without any further treatment.

2.3.8 Metabolite analysis

Consumption of glucose and concentration of main metabolites, lactic and acetic acids, were determined by HPLC (Merck Hitachi, Darmstadt, Germany). Samples were diluted in MilliQ water and filtered through a 0.45 µm nylon membrane filter (Infochroma AG, Zug, Switzerland). Separation was carried out in an Aminex HPX-87-H column (Bio-Rad Laboratories, Reinach, Switzerland) with 10 mM H$_2$SO$_4$ as mobile phase at a flow rate of 0.6 ml/min and a temperature of 40 °C. Compounds were detected using a refractive index detector (Merck Hitachi, Darmstadt, Germany). Reported data are means of duplicate analysis.

2.3.9 Fatty acid analysis

The fatty acid composition of cell membrane was determined by gas chromatography at the DSMZ external service (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) using the Microbial Identification System (MIDI, Newark, DE; http://www.midi-inc.com). Frozen cell samples of end exponential growth phase cells produced with batch cultures and from R2 of ICC2 at day 4, 12 and 20 were thawed at room temperature and washed (6,000 × g for 3 min) with PBSC and pellets were frozen using liquid nitrogen. Fatty acids were converted to methyl esters and extracted in a five-step procedure according to the DSMZ protocols. Analyzes were performed in duplicate.
2.3.10 **Heat test**

Heat survival tests were carried out according to Simpson *et al.* (2005) with some modifications. Briefly, frozen cell samples of ICC2 were thawed at room temperature and centrifuged at 6,000 × g for 2 minutes. Supernatant was discarded and pellet resuspended in the same volume of fresh MRSC and incubated for 4 h at 37 °C to reactivate cells. This treatment was selected based on preliminary screening tests to obtain sufficient cell activity for RNA degradation. Cell suspensions were split in 1 ml aliquots and mixed with either 9 ml pre-warmed MRSC at 56 °C or room temperature (control) and incubated in a water bath at the corresponding temperature for 30 min. After the stress, cells were immediately cooled to room temperature in a water bath for three minutes, washed in fresh MRSC and pellets suspended in 1 ml MRSC. To quantify viable cells, 30 µl of cell solution was applied to a dilution row in PBSC and appropriate dilutions were plated on MRSC agar.

To enumerate viable cells from macroscopic aggregates containing samples with qRT-PCR, 0.5 ml of the suspended cells solution was shock frozen in liquid nitrogen and stored at -80 °C for RNA-extraction and qRT-PCR analysis as described above. For all tests, controls consisted of cells produced during batch cultures. Experiments were performed in duplicate.

2.3.11 **Bile salt test**

The effects of bile salts were tested as previously described by Saarela *et al.* (2004) with some modifications. Samples were prepared as for the heat test. After a reactivation step at 37 °C for 4 h, cell samples from ICC1 were split in aliquots of 1 ml and immediately added to 9 ml 1.5 % (w/v) porcine bile salt (Sigma-Aldrich) dissolved in PBSC or in 9 ml PBSC without bile salts and incubated for 17 min at 37 °C. After the stress, cells were cooled to room temperature in a water bath.
bath for three minutes and washed twice in fresh MRSC to remove bile salts. To quantify viable cells, 30 µl of cell solution was applied to a dilution row in PBSC and appropriate dilutions were plated on MRSC agar.

Bile tolerance of samples from ICC2 was evaluated as described above, but lethal bile treatment was carried out with 1.0 % (w/v) bile solution for 12 min at 37 °C. Less restrictive stress conditions were chosen, because the detection limit of the qRT-PCR method (ca. $4.4 \times 10^4$ CFU/ml) was higher than for plate counts and could not detect viable after bile stress at 1.5 % (w/v) for 20 min of control cells. Survival was determined with standard plate counts and qRT-PCR as for the heat test. Experiments were performed in duplicate.

### 2.3.12 Tolerance to antibiotics

Antibiograms were performed using an antimicrobial disc susceptibility assay according to the National Commitee for Clinical Laboratory Standards (1991) with some modifications. Cell samples were diluted in MRSC to an OD$_{600}$ of 0.0125 and streaked out with a cotton tip on MRSC agar plates. Cells from IC cultures were only tested before formation of macroscopic aggregates because OD values of samples containing cell clusters could not be accurately measured and were not representative of cell counts. Discs of 6 mm diameter containing antibiotics were then placed on agar plates. The used antibiotic-discs were: Penicillin G (P 10), Norfloxacine (NOR 10), Nalidixic acid (NA 30), Ampicilline (AM 10), Cefotaxime (CTX 30), Erythromicin (E 15), Gentamicin (GM 10), Tetracycline (TE 30), Vancomicin (VA 30), Chloramphenicol (C 30) (BioMérieux Suisse SA, Geneva, Switzerland) and Neomicin (N 10), Bacitracin (B 10), Teicoplanin (TEC 30) (Oxoid AG, Switzerland)). Plates were incubated
anaerobically at 37 °C for 48 h and the inhibition zone was measured in mm. Experiments were performed in duplicate.

2.3.13 Statistical analyzes

Means of log\textsubscript{10}-transformed cell counts for heat- and bile tests as well as inhibition diameters of antibiograms and molecular acetate /lactate ratios were compared with Student’s \textit{t}-test using JMP In Software version 6.0 (SAS Institute Inc., Cary, NC) with a level of significance at 0.05.

2.4 Results

Two continuous IC cultures were carried out in a two-stage fermentation system (Figure 6) for 20 days and effluents of both fermentations and reactors were tested for cell morphology, cell and metabolites concentration, and cell survival to lethal heat and bile salt stresses to assess the effects of immobilization in combination with continuous culture time and different glucose concentrations.

2.4.1 Biomass production

Continuous culture of immobilized \textit{B. longum} NCC2705 induced formation of large cell aggregates in both reactors of ICC1 after 12 days of fermentation and to an even higher extent in ICC2 where macroscopic aggregates of ca. 0.5 – 1 mm diameter were observed after only 9 days of continuous culture (Figure 7).
Chapter 2 - Immobilized cells

Figure 7  Optical micrographs of fresh cells collected from fermented broth (1000 × magnification) of *B. longum* NCC2705 produced in batch culture (A) and in ICC2 from day 8 (B), day 12 (C) and day 20 (D).

Free cell population for ICC1 with limiting glucose tested in the fermented broth medium with plate counts were very similar in both reactors and decreased progressively with culture time from $1.4 \pm 0.1 \times 10^9$ to $3.4 \pm 0.7 \times 10^8$ CFU/ml between day 2 and 20 (Figure 8). Cell dry weight (CDW) of ICC1 also decreased progressively but to a lower extent with time in R1 from 1.9 at day 4 to 1.3 g/l at day 20, but remained constant in R2 at 1.7 ± 0.2 g/l throughout the entire 20 days (Figure 8). High maximal volumetric productivities were reached after 2 days of culture, with $6.2 \times 10^{11}$ for R1 and $2.5 \times 10^{11}$ CFU/l h for the two stage culture (Table 4).

In ICC2 operated with glucose supplementation, populations of both reactors tested with plate counts showed large fluctuations with fermentation time. From day 1 to 7 cell counts increased steadily from $1.2 \times 10^7$ to $1.0 \times 10^9$ and from $1.7 \times 10^7$ to $2.3 \times 10^9$ CFU/ml for R1 and R2, respectively (Figure 8). After detection of macroscopic cell aggregates at day 9, cell counts decreased continuously until the end of fermentation to $2.4 \times 10^9$ and $6.3 \times 10^8$ CFU/ml for R1 and R2, respectively (Figure 8). In contrast, viable cell concentration determined for R2 with aggregation independent qRT-PCR method increased steadily from $1.1 \pm 0.4$ to $8.6 \pm 4.4 \times 10^9$ CFU/ml between day 4 and 12 and remained stable afterwards (Figure 8 B). Similarly, CDW increased steadily from 0.3 to 2.7 g/l and from 0.3 to 4.6 g/l in R1 and R2, between day 2 and 20, respectively (Figure 8). CDW correlated well with viable cell counts tested with qRT-PCR but
not with plate counts. Maximal volumetric productivities reached $5.9 \times 10^{11}$ for R1 and $4.2 \times 10^{11}$ for the two stage culture determined by plate counts or $1.5 \times 10^{12}$ CFU/l h for the two stage culture determined with qRT-PCR method (Table 4).

The maximal cell concentration for batch cultures was $1.6 \pm 1.1 \times 10^9$ CFU/ml after 12.5 h growth in MRSC at an $OD_{600}$ of 5.7 ± 0.4 (early stationary growth phase) corresponding to a volumetric productivity of $1.2 \times 10^{11}$ CFU/l h (Table 4).

**Table 4** Maximal cell counts and volumetric productivities of *B. longum* NCC2705 in different fermentation systems. Values obtained by plate counts or by qRT-PCR method.

<table>
<thead>
<tr>
<th>Fermentation systems</th>
<th>Fermentation time</th>
<th>Dilution rate ((h^{-1}))</th>
<th>Cell counts ((CFU/ml))</th>
<th>max. vol. productivity ((CFU/l h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>13.5 h</td>
<td>-</td>
<td>$1.6 \times 10^9$</td>
<td>$1.2 \times 10^{11}$</td>
</tr>
<tr>
<td>ICC1 R1</td>
<td>2 days</td>
<td>2.25</td>
<td>$1.4 \times 10^9$</td>
<td>$6.2 \times 10^{11}$</td>
</tr>
<tr>
<td>ICC1 R1 + R2</td>
<td>2 days</td>
<td>0.18</td>
<td>$1.4 \times 10^9$</td>
<td>$2.5 \times 10^{11}$</td>
</tr>
<tr>
<td>ICC2 R1</td>
<td>6 days</td>
<td>2.25</td>
<td>$1.3 \times 10^9$</td>
<td>$5.9 \times 10^{11}$</td>
</tr>
<tr>
<td>ICC2 R1 + R2*</td>
<td>7 days</td>
<td>0.18</td>
<td>$2.3 \times 10^9$</td>
<td>$4.2 \times 10^{11}$</td>
</tr>
<tr>
<td></td>
<td>12 days (qRT-PCR)</td>
<td>( (7.9 \times 10^9) )</td>
<td>( (1.5 \times 10^{12}) )</td>
<td></td>
</tr>
<tr>
<td>Continuous IC(^1)</td>
<td>n.i.</td>
<td>0.5</td>
<td>$4.9 \times 10^9$</td>
<td>$2.5 \times 10^{12}$</td>
</tr>
<tr>
<td>Free cell continuous(^2)</td>
<td>105 h</td>
<td>0.1</td>
<td>$1.1 \times 10^9$</td>
<td>$1.1 \times 10^{11}$</td>
</tr>
</tbody>
</table>

* values in brackets from qRT-PCR
\(^1\) for *B. longum* ATCC15707 in Doleyres et al. (2002)
\(^2\) Mozzetti (2009)
Figure 8  Viable cell concentrations and cell dry weight (CDW) in samples from R1 (A) and R2 (B) during continuous immobilized cell cultures. Cell concentrations determined by plate counts (full line, □, ■) or by qRT-PCR (semi dashed line, ●) and cell dry weight (dashed line, Δ, ▲). Data from ICC1 (open symbols) and ICC2 (solid symbols) are means of two determinations.
2.4.2 Metabolic Activity

Glucose consumption in R1 of ICC1 was stable throughout the entire fermentation at 12.5 ± 0.2 g/l and resulted in almost no sugar in the effluent of R1 at a mean residual concentration of 1.1 ± 0.2 g/l (Figure 9). Lactic and acetic acid production were stable during 20 days, with 5.4 ± 0.4 and 6.2 ± 0.6 for R1 and 0.3 ± 0.5 and 0.8 ± 0.9 g/l for R2, respectively. Therefore the molar ratio of main metabolites acetic and lactic acids in R1 remained also constant during the entire fermentation at 1.72 ± 0.07. The low sugar concentration in the effluent from R1 of ICC1 caused starving conditions in R2 and hence formation of metabolites was not relevant.

In ICC2, glucose consumption varied with time in both reactors. In R1, glucose consumption increased steadily from 16.0 ± 1.3 at day 6 to 39.7 ± 0.2 g/l at day 13 after an adaptation phase during the first five days. Afterwards, consumption decreased to reach 29.9 ± 0.6 g/l at day 20. In contrast, glucose consumption in R2 increased steadily between day 1 and 7 from 1.8 ± 0.0 to 23.0 ± 0.5 g/l, then decreased sharply to 4.1 ± 0.2 g/l at day 12. From day 13 until the end of fermentation, consumption increased again to reach 19.5 ± 0.0 g/l. Residual glucose concentrations in ICC2 were never lower than 13.1 ± 0.2 and 1.8 ± 0.1 in R1 and R2, respectively, indicating no sugar limitation. The acetic and lactic acid production in R1 increased from 7.0 ± 0.1 and 5.0 ± 0.1 at day 3 to 15.3 ± 0.2 and 14.3 ± 0.3, respectively, and then decreased until the end of fermentation to 11.4 ± 0.4 and 11.0 ± 0.4 g/l, respectively. The production of acetic and lactic acid in R2 varied like the sugar consumption and ranged between a maximum of 9.4 ± 0.9 and 7.9 ± 0.8 at day 7 and a minimal value of 1.7 ± 1.1 and 0.9 ± 1.0 g/l at day 12, respectively. The molar ratio of acetic to lactic acids showed a slight but significant decrease in R1 after an adaptation phase in the first 3 days, from 1.75 at day 4 to 1.56 at day 20.
R2 showed large fluctuations over time in the range of 1.58 to 2.85, mirroring glucose consumption (Figure 9 B).

Glucose in batch culture was entirely consumed when cells reached early stationary growth phase after 12.5 h growth. The levels of acetic and lactic acid reached the maximum at mid stationary growth phase (16 h) with 7.3 ± 0.0 and 5.6 ± 0.0 g/l, respectively, corresponding to a molar ratio of acetic to lactic acids of 1.93.
Figure 9  Consumption of glucose and molar ratio of acetic to lactic acid in samples from R1 (A) and R2 (B) during continuous immobilized cell cultures. Glucose consumption (full line; □, ■) and molar ratio of acetate and lactate (dashed line; Δ, ▲). Data from ICC1 (open symbols) and ICC2 (solid symbols) are means of two determinations.
2.4.3 Cell sensitivity to heat and porcine bile salts

Cell tolerance to porcine bile salt stress was first tested by exposing cells from ICC1 and control batch for 20 min to 1.5 % (w/v) porcine bile salt solution and measured by plate count enumeration. Samples from ICC1 at day 4, 12 and 20 showed significantly less loss of viable cells of $3.5 \pm 0.4$, $2.8 \pm 0.1$ and $3.0 \pm 0.1$ for R1 and $2.6 \pm 0.3$, $2.6 \pm 0.4$ and $1.8 \pm 0.2$ log\textsubscript{10} CFU/ml for R2, respectively, compared to batch stationary phase cells with $5.7 \pm 1.2$ log\textsubscript{10} loss of CFU/ml (Table 5). Indeed, the viable cell counts of batch control samples after exposure to 1.5 % (w/v) bile salt solution were too low to be detected with the qRT-PCR method (detection limit ca. $4.5 \times 10^4$ CFU/ml) and hence the conditions for the bile assay were set at 1.0 % (w/v) bile salt solution to test samples from ICC2 and batch control cells. Viability loss of ICC2 cells was tested for cells from R2 at day 4, 12 and 20, using plate counts and qRT-PCR method. The bile assay was carried out for 15 min at 1.0 % (w/v) bile salt solution, Viability loss of cell samples evaluated with plate counts was $3.3 \pm 0.0$, $1.7 \pm 0.1$, $3.0 \pm 0.2$ log\textsubscript{10} CFU/ml for cells from R2 day 4, 12, 20 and significantly smaller than $4.0 \pm 0.2$ log\textsubscript{10} CFU/ml of batch control cells. When tested with qRT-PCR, viability losses for cells from day 4 and 20 were $0.6 \pm 0.2$ and $0.5 \pm 0.2$ log\textsubscript{10} CFU/ml, respectively, and also significantly smaller than $1.0 \pm 0.1$ log\textsubscript{10} CFU/ml for batch control cells, whereas no significant difference was detected for cells from day 12 (Table 5).

Heat tolerance was tested by heating cell samples from ICC2 R2 at 56 °C for 30 min. Viability losses tested with plate counts of cells collected at day 4, 12 and 20 range between $4.1 - 3.4$ log\textsubscript{10} CFU/ml and were not significantly different to $3.4 \pm 0.4$ log\textsubscript{10} CFU/ml of batch control cells. Similarly, no significant differences between cells from continuous and batch were observed using qRT-PCR method (Table 5).
### Table 5

Cell viability loss after exposure to porcine bile salt and heat lethal stresses of cells from batch, and continuous immobilized cell cultures at day 4, 12 and 20. Values are given as loss of log\(_{10}\) (CFU/ml) determined by plate counts or qRT-PCR. Data are means of two independent repetitions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Detection method</th>
<th>Batch</th>
<th>ICC1*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>day 4</td>
<td>Day 12</td>
</tr>
<tr>
<td>20 min in 1.5% porcine bile</td>
<td>Plate counts</td>
<td>5.7 ± 1.2(^a)</td>
<td>3.5 ± 0.4(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R1</td>
<td>R2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.6 ± 0.3(^b)</td>
<td>2.6 ± 0.4(^b)</td>
</tr>
<tr>
<td>30 min at 56°C</td>
<td>Plate counts</td>
<td>3.4 ± 0.4(^a)</td>
<td>4.1 ± 0.1(^b)</td>
</tr>
<tr>
<td></td>
<td>qRT-PCR</td>
<td>2.3 ± 0.3(^a)</td>
<td>2.5 ± 0.2(^a)</td>
</tr>
<tr>
<td>15 min in 1% porcine bile §</td>
<td>Plate counts</td>
<td>4.0 ± 0.2(^a)</td>
<td>3.3 ± 0.0(^b)</td>
</tr>
<tr>
<td></td>
<td>qRT-PCR</td>
<td>1.0 ± 0.1(^a)</td>
<td>0.6 ± 0.2(^b)</td>
</tr>
</tbody>
</table>

Different letters on each line (p ≤ 0.05).
* Values from R1 and R2

### 2.4.4 Tolerance to antibiotics

Tolerances to antibiotics were tested using disc-assay and only applied to samples from continuous cultures before formation of aggregates. No inhibition zone was observed for cells from ICC1 and 2 and batch cultures to norfloxacin and nalidixic acid (Table 6). Cells from ICC1 collected at day 2 and 10 showed significant increased tolerance to aminoglycosidic gentamicin and neomycin (p ≤ 0.05) compared to batch control cells, and a slight but not significant increased tolerance with culture time for both antibiotics (Table 6).

A significant increase in tolerance of cells from ICC2 was detected between day 2 and 8 for penicillin, erythromycin, gentamicin, tetracycline, vancomycin, neomycin, bacitracin and
teicoplanin. Furthermore, cells collected at day 8 showed significantly smaller diameters ($p \leq 0.05$) for gentamicin and neomycin, compared to cells from control batch (Table 6).

### Table 6

Inhibition diameters measured by the disc assay method of different antibiotics on cells from control batch cultures and produced by continuous immobilized cell cultures ICC1 and ICC 2 tested at different days of fermentation before formation of macroscopic cell aggregates. Values are means of two repetitions.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Batch</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICC1</td>
<td>ICC2</td>
<td>ICC1</td>
<td>ICC2</td>
<td>ICC1</td>
<td>ICC2</td>
<td>ICC1</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 10</td>
<td>Day 2</td>
<td>Day 8</td>
<td>Day 2</td>
<td>Day 8</td>
<td>Day 2</td>
</tr>
<tr>
<td>Penicillin G [10]</td>
<td>42.0 ± 1.3a</td>
<td>-</td>
<td>-</td>
<td>52.5 ± 0.7b</td>
<td>47.5 ± 0.7c</td>
<td>42.0 ± 1.3a</td>
<td>-</td>
</tr>
<tr>
<td>Norfloxocine [10]</td>
<td>6.0 ± 0.0+a</td>
<td>-</td>
<td>-</td>
<td>6.0 ± 0.0+a</td>
<td>6.0 ± 0.0+a</td>
<td>6.0 ± 0.0+a</td>
<td>6.0 ± 0.0+a</td>
</tr>
<tr>
<td>Nalidixic acid [30]</td>
<td>6.0 ± 0.0+a</td>
<td>-</td>
<td>-</td>
<td>6.0 ± 0.0+a</td>
<td>6.0 ± 0.0+a</td>
<td>6.0 ± 0.0+a</td>
<td>6.0 ± 0.0+a</td>
</tr>
<tr>
<td>Ampicillin [10]</td>
<td>41.0 ± 2.3a</td>
<td>-</td>
<td>-</td>
<td>45.5 ± 3.5b</td>
<td>37.5 ± 4.9a</td>
<td>41.0 ± 2.3a</td>
<td>-</td>
</tr>
<tr>
<td>Cefotaxime [30]</td>
<td>41.0 ± 2.9a</td>
<td>40.0 ± 1.3a</td>
<td>38.8 ± 1.0a</td>
<td>45.5 ± 3.5b</td>
<td>37.5 ± 4.9a</td>
<td>45.5 ± 3.5b</td>
<td>37.5 ± 4.9a</td>
</tr>
<tr>
<td>Erythromycin [15]</td>
<td>45.0 ± 1.4a</td>
<td>-</td>
<td>-</td>
<td>51.5 ± 2.1b</td>
<td>44.5 ± 0.7a</td>
<td>45.0 ± 1.4a</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin [10]</td>
<td>16.0 ± 1.3a</td>
<td>10.2 ± 2.4b</td>
<td>9.3 ± 2.3b</td>
<td>23.0 ± 1.4c</td>
<td>8.0 ± 2.8b</td>
<td>16.0 ± 1.3a</td>
<td>10.2 ± 2.4b</td>
</tr>
<tr>
<td>Tetracycline [30]</td>
<td>41.0 ± 1.3a</td>
<td>-</td>
<td>-</td>
<td>46.5 ± 3.5b</td>
<td>41.0 ± 0.0a</td>
<td>46.5 ± 3.5b</td>
<td>41.0 ± 0.0a</td>
</tr>
<tr>
<td>Vancomycin [30]</td>
<td>31.0 ± 1.0a</td>
<td>-</td>
<td>-</td>
<td>38.5 ± 0.7b</td>
<td>32.0 ± 0.0a</td>
<td>38.5 ± 0.7b</td>
<td>32.0 ± 0.0a</td>
</tr>
<tr>
<td>Chloramph. [30]</td>
<td>44.0 ± 1.3a</td>
<td>-</td>
<td>-</td>
<td>50.0 ± 4.2b</td>
<td>46.0 ± 0.0ab</td>
<td>44.0 ± 1.3a</td>
<td>-</td>
</tr>
<tr>
<td>Neomycin[10]</td>
<td>14.0 ± 1.3b</td>
<td>9.4 ± 1.7b</td>
<td>8.6 ± 1.5b</td>
<td>16.5 ± 0.7a</td>
<td>6.0 ± 0.0b</td>
<td>14.0 ± 1.3b</td>
<td>9.4 ± 1.7b</td>
</tr>
<tr>
<td>Bacitracin[10]</td>
<td>23.0 ± 2.2a</td>
<td>-</td>
<td>-</td>
<td>31.0 ± 2.8b</td>
<td>24.0 ± 0.0a</td>
<td>23.0 ± 2.2a</td>
<td>-</td>
</tr>
<tr>
<td>Teicoplanin [30]</td>
<td>30.0 ± 0.0a</td>
<td>-</td>
<td>-</td>
<td>36.5 ± 2.1b</td>
<td>29.0 ± 1.4a</td>
<td>30.0 ± 0.0a</td>
<td>-</td>
</tr>
</tbody>
</table>

*6 mm diameter refers to no inhibition (diameter of antibiotic disc).
- : not tested
Different letters on each line ($p \leq 0.05$).

#### 2.4.5 Cell membrane fatty acid composition

Fatty acid composition of membranes of cells produced in ICC2 exhibited no differences between R1 and R2 (data not shown) but showed large fluctuations compared to membranes of batch culture cells (Table 7). Major changes in fatty acid composition were observed in saturated 14 : 0 and 16 : 0, as well as in unsaturated 18 : 1w9c fatty acids which account for more than 60 % of the total fatty acids of membranes from cells of all tested samples (Table 7). The ratio of
unsaturated to saturated fatty acids (USFA/SFA) of cells collected during continuous IC culture was $0.58 \pm 0.09$ and significantly lower than $1.74 \pm 0.00$ for cells from free cells batch culture.

**Table 7**  Fatty acid membrane composition in % of total fatty acids of cells from ICC2 collected at day 4, 12, 20 and batch culture cells. Data are means of two determinations.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Batch</th>
<th>ICC2 R2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 12</td>
</tr>
<tr>
<td>12:0</td>
<td>0.33 ± 0.01</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>14:0*</td>
<td>2.91 ± 0.04</td>
<td>14.36 ± 0.82</td>
</tr>
<tr>
<td>16:0</td>
<td>17.57 ± 0.08</td>
<td>27.73 ± 2.81</td>
</tr>
<tr>
<td>18:0</td>
<td>10.16 ± 0.06</td>
<td>6.94 ± 0.51</td>
</tr>
<tr>
<td>19:0 cyclo*</td>
<td>1.95 ± 0.00</td>
<td>10.34 ± 0.39</td>
</tr>
<tr>
<td>19 cycloprop</td>
<td>1.36 ± 0.04</td>
<td>6.01 ± 0.90</td>
</tr>
<tr>
<td>13:1 none</td>
<td>0.21 ± 0.01</td>
<td>3.25 ± 0.43</td>
</tr>
<tr>
<td>16:1 w9c</td>
<td>0.42 ± 0.01</td>
<td>1.60 ± 0.02</td>
</tr>
<tr>
<td>17:1 w9c</td>
<td>5.59 ± 0.10</td>
<td>1.13 ± 0.04</td>
</tr>
<tr>
<td>18:1 w9c*</td>
<td>50.25 ± 0.13</td>
<td>18.89 ± 0.43</td>
</tr>
<tr>
<td>18:1 w7c*</td>
<td>5.70 ± 0.03</td>
<td>1.71 ± 1.55</td>
</tr>
<tr>
<td>18:1 w6c</td>
<td>n.d.</td>
<td>3.48 ± 0.83</td>
</tr>
<tr>
<td>18:0 OH</td>
<td>5.10 ± 0.03</td>
<td>2.91 ± 1.20</td>
</tr>
<tr>
<td>Total BCFA (ISO)</td>
<td>1.46 ± 0.01</td>
<td>0.73 ± 0.23</td>
</tr>
<tr>
<td>Total DMA</td>
<td>32.63 ± 0.01</td>
<td>25.15 ± 0.89</td>
</tr>
<tr>
<td>USFA</td>
<td>62.17 ± 0.08</td>
<td>30.05 ± 1.33</td>
</tr>
<tr>
<td>SFA</td>
<td>35.72 ± 0.07</td>
<td>66.88 ± 0.73</td>
</tr>
<tr>
<td>USFA/SFA</td>
<td>1.74 ± 0.00</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>Labeled</td>
<td>97.89 ± 0.15</td>
<td>96.93 ± 0.63</td>
</tr>
<tr>
<td>Not labeled</td>
<td>2.12 ± 0.15</td>
<td>3.07 ± 0.63</td>
</tr>
</tbody>
</table>

* Fatty acids also including DMA forms.

n.d., not detected; USFA, unsaturated fatty acids; SFA, saturated fatty acids; BCFA, branched chain fatty acids; DMA, dimethyl acetal fatty acids
2.5 Discussion

Immobilized cell technology (ICT) in combination with continuous culture has previously been suggested for the production of starter or probiotic cultures with enhanced functional and technological properties and high volumetric productivities (Doleyres and Lacroix, 2005). In the present study, two continuous IC cultures were carried out for 20 days with and without glucose limiting conditions. The supplementation of additional glucose resulted in a high maximal cell production of $7.9 \times 10^9$ CFU/ml and volumetric productivity of $1.5 \times 10^{12}$ CFU/l h for the two stage culture measured with qRT-PCR was reached in ICC2 at day 12. This value is similar to that reported by Doleyres et al. 2002 during continuous culture with immobilized B. longum ATCC15707 in MRS medium supplemented with whey permeate at a dilution rate of 0.5 h$^{-1}$ and approximately ten fold higher than that of continuous culture with free cells of B. longum NCC2705 in MRSC operated at a dilution rate of 0.1 h$^{-1}$ as reported in a by Mozzetti (2009) and controlled batch cultures (Table 4).

Formation of large cell aggregates was observed in both continuous cultures leading to considerable underestimation of cell concentration with plate counts. Indeed, accurate viable cell enumeration of B. longum NCC2705 using the recently developed qRT-PCR method which is independent of cell aggregates (see Chapter 3) detected higher viable cell counts compared to traditional plate counts. Extensive formation of cell aggregates has already been reported during continuous cultures with immobilized lactobacilli (Bergmaier et al., 2005; Koch, 2006), whereas no cell aggregates were reported for the B. longum NCC2705 grown for more than 8 days in continuous mode with free cells in MRS (Mozzetti, 2009). Aggregation of bacterial cells can be induced by changing environmental factors for instance pH, level of divalent cations in the
medium and/or nutrients availability (Koop et al., 1989; Canzi et al., 2005, De Schryver et al., 2008).

In this study, formation and variation of size of cell aggregates in both IC cultures may have different origins. Although the pH in the bioreactors was constant at 6.0 during all fermentations (ICC and batch), the effect of acidic conditions in gel beads could be involved in cell aggregation. Indeed, steep pH gradients can develop within gel beads (Masson et al., 1994; Schepers et al., 2006). Larger size of aggregates in ICC2 may be a result of the supplementation of growth medium with CaCl$_2$ because Ca$^{2+}$ ions in the growth medium were previously shown to induce auto-aggregation in bifidobacteria (Ibrahim et al., 2005). Divalent cations may act as a bridging compound for negatively charged functional groups on the cell surface and may promote adhesion between bacterial cells (Higgins and Novak, 1997). Another factor affecting size of aggregates is the supplementation of growth medium with additional glucose. Higher sugar concentration in fermentation broth yielded higher biomass concentration and consequently more cell growth and larger sized cell aggregates. The sugar limiting conditions of ICC1 may also explain a metabolic shift towards production of acetic acid resulting in elevated molecular ratio of acetic and lactic acid. Enhanced acetic acid production was shown to improve the ATP-yield in B. animalis (Ruas-Madiedo et al., 2005), thereby generating higher energy resources for starving cells from equal amount of glucose. The molar ratio in batch cultures was higher than in continuous cultures, but comparison of the ratio is limited due to metabolite accumulation in batch fermentation systems.

Physicochemical characteristics of the cell surface such as hydrophobicity may also affect auto-aggregation and adhesion of bacterial cells to different surfaces (Perez et al., 1998; Del Re et al., 2000; Zavaglia et al., 2002). Changes in membrane characteristics upon growth in adverse micro-environment created within gel structures were already reported for Lactococcus lactis ssp. lactis
SL03 and *Lactococcus lactis* ssp. *cremoris* SC09 (Trauth *et al.*, 2001) or resulted in lower USFA/SFA ratio in fatty acid composition of membranes of immobilized *Saccharomyces cerevisiae* (Jirku *et al.*, 1999). In the present study, immobilized *B. longum* exhibited notable modifications in fatty acid profiles (e.g. lower USFA/SFA ratio) of cell membranes compared to free cells grown in batch cultures. Alterations in membrane composition may occur as a result of an adaptation to changing environment and can lead to changes in membrane hydrophobicity as shown for starved or acid-adapted *Listeria innocua* cells (Moorman *et al.*, 2008).

The observed changes in membrane composition of cells released from gel beads may also play an important role in their improved resistance to bile salts and the aminoglycosidic antibiotics gentamycin and neomycin. A similar decrease of the ratio of unsaturated to saturated fatty acids of cell membrane from IC cultures was reported for a bile adapted mutant strain of *B. animalis* compared to its mother strain (Ruiz *et al.*, 2007). These changes might be involved in restricted diffusion of bile salts into the cytoplasm (Begley *et al.*, 2005). The higher cell loss tested with aggregated cells and plate counts compared to qRT-PCR were not expected because in theory one viable cell in aggregate can give a colony, leading to an overestimation of cell survival after the bile test. The observed difference in viability-loss between plate count and qRT-PCR method can be a result of a bile induced sublethal injury within the *Bifidobacterium* population, possibly through a reversible and transient membrane permeabilization which resulted in a loss of viability, as defined by plate counts, but these cells could regain growth after being sorted and resuscitated as reported by Ben-Amor *et al.* (2002).

Modifications in the membrane properties might also prevent passive diffusion of aminoglycosides into the cell (Bryan *et al.*, 1984). The integrity of membranes of *B. longum* was shown to be involved in the tolerance to aminoglycosides, where susceptibility to
aminoglycosides increased when cells were previously subjected to membrane corrupting oxgall or H$_2$O$_2$ stress (Kheadr et al., 2007).

Doleyres et al. (2004a) reported a progressive increase of survival of *B. longum* ATCC 15707 to simulated intestinal conditions and tolerance to nisin Z with fermentation time induced by cell immobilization in continuous co-culture with *Lc. lactis* MD. The increase of bile tolerance with time correlated with increased tolerance to nisin Z, which targets bacterial cell membranes. These findings support the observations in this study of increased tolerance to bile salts due to membrane modifications induced by IC and continuous culture. The effect of culture age on stress tolerance can be strain dependent and/or may originate from an adaptation process with fermentation time of *B. longum* to restrictive environments produced by the competitive *Lc. lactis* strain (Doleyres et al., 2004a). In a recent study, a co-culture of *B. longum* and *B. breve* was cultivated in a compartmentalized system where the cells grew separately but the supernatants were mixed together (Ruiz et al., 2009). The authors reported an increased expression of stress response proteins such as chaperones in *B. breve* which may protect bacterial cells from environmental stresses.

Cells from ICC2 did not exhibit detectable changes in tolerance to heat compared to batch cells. Although, changes in membrane composition can influence bacterial membrane fluidity and hence cell tolerance to heat (Guillot et al., 2000; Denich et al., 2003), the increase in shorter fatty acids (acyl-chains of less than 18 carbons) and the decrease of unsaturated fatty acids in membranes of cells from ICC2 did not affect survival to lethal heat stress. It is noteworthy to mention that preliminary results from gene expression analyzes of cells from ICC2 compared with control batch cells showed that genes coding for stress related proteins were not differentially expressed as reported for heat stressed *B. longum* NCC2705 cells (Rezzonico et al., 2007).
The use of aggregated cells in probiotic food industries is still unfavorable in product applications due to underestimation of viable cell counts with standard plate counts that is the standard method to verify product quality (see Chapter 3). However, the ability to form aggregates could be a beneficial functionality for probiotic strains. Indeed, aggregation ability of *Bifidobacterium* as well as *Lactobacillus* cells has been associated with enhanced adhesion to epithelial cells from human or pigs (Del Re et al., 2000; Kos et al., 2003), coaggregation with pathogenic bacteria (Schachtsiek et al., 2004; Collado et al., 2007) and improved protective effect against colitis in mice (Castagliuolo et al., 2005). Further experiments are required to test whether observed morphological and physiological changes of cells from IC cultures affect survival during GIT transit and adhesion-ability to epithelial cells.
3 Development of a real-time RT-PCR method for enumeration of viable *Bifidobacterium longum* cells in different morphologies

Data presented in this chapter were published in 2009 in Food Microbiology with the following authors: Reimann S., Grattepanche F., Rezzonico E., and Lacroix C.
3.1 Abstract

Viability of probiotic bacteria is traditionally assessed by plate counting which has several limitations, including underestimation of cells in aggregates or chains morphology. We describe a quantitative PCR (qPCR)-based method for an accurate enumeration of viable cells of *Bifidobacterium longum* NCC2705 exhibiting different morphologies by measuring the mRNA levels of *cysB* and *purB*, two constitutively expressed housekeeping genes. Three primer-sets targeting short fragments of 57-bp of *cysS* and *purB* and one 400-bp fragment of *purB* were used. Cell quantification of serially diluted samples showed a good correlation coefficient of $R^2$ 0.984 ± 0.003 between plate counts and qRT-PCR for all tested primer sets. Loss of viable cells exposed to a lethal heat stress (56 °C, 10, 20 and 30 min) was estimated by qRT-PCR and plate count. No significant difference was observed using qRT-PCR targeting the 400-bp fragment of *purB* compared to plate count indicating that this fragment is a suitable marker of cell viability. In contrast, the use of the 57-bp fragments led to a significant overestimation of viable cell counts (18 ± 3 and 7 ± 2 fold for *cysB* and *purB*, respectively). Decay of the mRNA fragments was studied by treatment of growing cells with rifampicin prior qRT-PCR. The 400-bp fragment of *purB* was faster degraded than the 57-bp fragments of *cysB* and *purB*. The 400-bp fragment of *purB* was further used to enumerate viable cells in aggregate state. Cell counts were more than $2 \log_{10}$ higher using the qRT-PCR method compared to plate count.

Growing interest in probiotic characteristics of aggregating bacteria cells make this technique a valuable tool to accurately quantify viable probiotic bacteria exhibiting heterogeneous morphology.
3.2 Introduction

Probiotics are defined as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2001). It is generally accepted that probiotic food products should contain a minimal level of viable cells of $10^6$ per gram or milliliter of product (Ouwehand et al., 1998), although this value is relative since beneficial effect depends on the strain and targeted health benefit (Lacroix and Yıldırım, 2007). Viability of probiotic bacteria is traditionally assessed by plate counting which has several limitations, including underestimation of cells in aggregates or chains morphology. Auto-aggregation trait of *Bifidobacterium longum* strains isolated from human gastric juice and intestine has been associated with their ability to adhere to epithelial cells (Del Re et al., 2000) which is considered as an important functionality of probiotic bacteria for some beneficial health effects such as to prevent attachment of pathogens to gut mucosa (Resta-Lenert and Barrett, 2003; Cesena et al., 2001; Castagliuolo et al., 2005).

However, bacterial cell aggregates lead to underestimation of cell counts with traditional, culture-dependent methods (Davey et al., 1977; Auty et al., 2001). Enumeration techniques with fluorescent stains targeting bacterial membrane potentials or enzymatic activities were proposed as alternative methods to plate counts (Breeuwer and Abee, 2000; Hewitt and Nebe-Von-Caron, 2001; Alakomi et al., 2005). The accuracy of these methods using fluorescence probes, generally combined with flow cytometry can be affected by clustering and chaining of cells (Wallner et al., 1995; Nebe-von-Caron et al., 2000; Bibiloni et al., 2001).

The use of real-time PCR techniques to quantify bifidobacteria and lactic acid bacteria by specific DNA or rRNA targeting showed good correlation with plate counts (Masco et al., 2007; Friedrich and Lenke, 2006; Matsuda et al., 2007). However, their use as accurate viability
markers is limited since rRNA and DNA can be detected hours or even days after cell death (Tolker-Nielsen et al., 1997; Sheridan et al., 1998; Hellyer et al., 1999; Aellen et al., 2006; Keer and Birch, 2003; Lahtinen et al., 2008). Molecules of mRNA in contrast have short half-life times ranging between 0.5 and 50 min (Belasco et al., 1986; Hellyer et al., 1999; Takayama and Kjelleberg, 2000) and are therefore a more suitable viability marker for bacterial cells. Yeasts and pathogenic bacteria were quantified using mRNA in combination with quantitative reverse transcription PCR (qRT-PCR) (Bleve et al., 2003; Jacobsen and Holben, 2007; Coutard et al., 2007; Gonzalez-Escalona et al., 2009). The use of instable mRNA as target molecule however requires special attention to minimize losses during RNA extraction and purification (Bustin 2002, Johnson et al., 2005).

In this study, we describe the development of a qRT-PCR-based method for the quantification of viable *B. longum* NCC2705 cells. Serial dilutions of bacterial culture aliquots were used to generate qRT-PCR calibration curves targeting three mRNA fragments (two short amplicons of 57-bp of *cysS* or *purB* and one of 400-bp of *purB*) and plotted against plate counts. Spiked internal reference mRNA was used to monitor mRNA loss during sample processing and thus to provide accurate mRNA comparison across different experimental conditions (Johnson et al., 2005). Stability of the three mRNA fragments was tested by comparing cell counts obtained by qRT-PCR and plate counts of culture exposed to lethal heat stress. Instability of the different mRNA fragments was also assessed by measuring mRNA decay after rifampicin treatment. The mRNA fragment selected was then used to estimate viable cell numbers in culture samples containing macroscopic cell aggregates.
3.3 Materials and Methods

3.3.1 Strain and growth conditions

Bifidobacterium longum NCC2705 (Nestlé Culture Collection, Nestlé Research Center, Lausanne, Switzerland) was grown at 37 °C in rubber-lid flasks containing 100 ml MRS broth (de Man et al., 1960) (BioLife, Milan, Italy) supplemented with 0.5 g/l L-cysteine (Sigma-Aldrich, Buchs, Switzerland) (MRSC) and inoculated at 2 % (v/v) with an overnight MRSC pre-culture. The headspace of flasks was flushed with CO₂ to ensure anaerobic conditions. Colony forming units (CFU) were determined by plating serial dilutions of culture aliquots in duplicate on MRSC agar plates 1.5 % (w/v) (Becton Dickinson, Basel, Switzerland) and incubating at 37 °C for 48 h in anaerobic jars containing atmosphere generation system packs (AnaeroGen, Oxoid, Pratteln, Switzerland).

3.3.2 Preparation of bacterial samples for calibration curves

Cultures were grown at 37 °C in a 2000 ml reactor (Bioengineering, Wald, Switzerland) containing 1800 ml MRSC inoculated at 0.5% (v/v) with pre-culture. pH was maintained at 6.0 by addition of NaOH (5 M), agitation rate was set at 200 rpm using a flat blade impeller and anaerobic conditions were maintained by flushing CO₂ in the head space of the reactor. Cells were harvested in end-exponential growth phase and centrifuged at 6,000 × g for 2 min. Pellets were suspended in an equal volume of a cryoprotectant solution composed of MRSC supplemented with glycerol 10% (w/v) (Fluka, Buchs, Switzerland), shock frozen in liquid nitrogen and stored at -80 °C before RNA extraction and plate counts. Standards with cell concentrations ranging from about 3.2 × 10⁴ to 4.7 × 10⁸ CFU/ml determined by plate counts were prepared by serial dilution in MRSC before RNA extraction.
3.3.3 Production of aggregated cells

*B. longum NCC2705* cells aggregates were produced during continuous culture with immobilized cells grown in a two-stage reactor system as described by Doleyres *et al.* (2004b), with some modifications. Briefly, the first reactor (R1) was a 500 ml glass reactor (Sixfors, Infors-HT, Bottmingen, Switzerland) with a working volume of 240 ml, containing 80 ml of gel beads pre-colonized with *B. longum NCC2705* as described by Cinquin *et al.* (2004) and stirred between 150 – 250 rpm. The second 2000 ml reactor (Bioengineering) was run in series at a volume of 1800 ml, agitated at 250 rpm and inoculated with cells produced in R1. CO$_2$ was injected in the headspace of the two reactors to maintain anaerobic conditions during culture. The continuous system was fed with MRSC medium supplemented with 1.5 g/l CaCl$_2$ (Sigma-Aldrich) and 40 g/l glucose at a flow rate of 360 ml/h. Continuous fermentation was run for 20 days at 37 °C and pH was maintained at pH 6.0 by addition of NaOH (5 M) in both reactors. Culture samples from the effluent of both reactors were periodically collected and processed as described above.

3.3.4 Heat stress

A 100-ml volume of MRSC was inoculated at 1% (v/v) with *B. longum NCC2705* and incubated in a rubber-lid flask at 37 °C for 16 h. Cells were collected by centrifugation at 6,000 × g for 2 min. Supernatant was discarded and pellet suspended in the same volume of fresh MRSC. Cell suspensions were split in aliquots of 1 ml and immediately added to 9 ml MRSC either pre-warmed at 56 °C for 10, 20 and 30 min or kept at room temperature. After heating, cells were immediately cooled to room temperature in a waterbath for 3 min, collected by centrifugation, washed and suspended in the initial volume of MRSC for plate counting. 0.5 ml of the cell
suspension was added to prepared screw cap tubes as described below and immediately frozen in liquid nitrogen for RNA extraction. Experiments were performed in triplicate.

3.3.5 Rifampicin test

MRSC (100 ml) in a rubber-lid flask was inoculated at 5% (v/v) with preculture and incubated at 37 °C until the culture reached mid-exponential phase (OD$_{600}$ 0.5 – 0.8). Rifampicin (Sigma-Aldrich) was then added to a final concentration of 400 µg/ml. Samples were taken 2.5, 7.5, 14, 20, 25 and 45 min after rifampicin-addition. Cells were collected by centrifugation, washed in fresh MRSC and suspended in the initial volume of MRSC for RNA extraction. The half-life time ($T_{\frac{1}{2}}$) of an mRNA was determined from the degradation rate constant ($k$) corresponding to the slope of a semi-logarithmic plot of mRNA amount as a function of time with the relation: $T_{\frac{1}{2}} = \ln2/k$. Experiments were performed in duplicate.

3.3.6 RNA isolation and reverse transcription reaction

RNA extraction was performed according to Stevens et al. (2008). Briefly, 0.5 ml of fresh or stored cell samples was centrifuged and the pellet was washed once in MRSC, transferred to 2-ml screw-cap tubes containing a mix of 500 mg glass beads of 100 µm diameter (Sartorius, Goettingen, Germany), 500 µl of a 1:1 solution of phenol/chloroform (Sigma-Aldrich), 30 µl of 10% (w/v) sodium dodecyl sulfate (Sigma-Aldrich) and 30 µl of 3 M sodium acetate (pH 5.2) and immediately frozen in liquid nitrogen. Frozen cells were then disrupted by bead-beating in a homogenizer (FastPrep FP 120, Q-Biogene, Carlsbad CA, USA), four times for 40 sec at speed 4.0 intercalated with cooling periods on ice. Afterwards, the mixtures were centrifuged (21,000 × g, 3 min at 4 °C) to remove glass beads and cell debris. The aqueous phase was transferred to a
new tube and traces of phenol were removed by extraction with pre-chilled chloroform. The resulting aqueous phase was mixed with an equal volume of 70%-ethanol and applied to RNA purification column using RNeasy Mini Kit (Qiagen, Basel, Switzerland). Further purification was performed according to the manufacturer’s protocol including on-column DNase I digestion for 30 min followed by elution in 100 µl H₂O. Total RNA quantity and purity was determined using Nanodrop ND-1000 (Peqlab, Erlangen, Germany).

Reverse transcription (RT) was performed using the high capacity cDNA reverse transcription commercial kit (Applied Biosystems, Rotkreuz, Switzerland) according to manufacturer’s protocol. A maximum amount of 100 ng of purified total RNA was applied per RT reaction. Reaction was carried out in a thermocycler (Biometra T-personal, Göttingen, Germany) using the following cycling conditions: 10 min at 25 °C, 120 min at 37 °C, 5 sec at 85 °C followed by a cooling step to 4 °C.

### 3.3.7 Determination of RNA recovery rate using internal reference

Luciferase mRNA (Promega, Dübendorf, Switzerland) was used as internal reference to estimate loss of RNA during extraction and purification procedures. 2.03 × 10⁸ copies of luciferase mRNA were added to the aqueous phase, after centrifugation of lysed cells. Copy numbers of luciferase mRNA added per volume were calculated using transcript sizes of 1.8 kb and an average molecular mass of 330 Da/nucleotide. The number of luciferase mRNA copies recovered from spiked samples after RNA extraction was determined with qRT-PCR and standard curves prepared from serial dilutions of luciferase mRNA. Recovery rate was evaluated by dividing the measured level by the initial amount of spiked reference mRNA and used as normalization factor to correct for loss of mRNA during the RNA isolation procedure.
3.3.8 Quantitative PCR amplification

Primers used for quantitative PCR amplification (Table 7) were designed with PrimerExpress software Version 3.0 (Applied Biosystems). Genes cysS and purB were selected for their constitutive expression under different culture conditions (Rezzonico et al., 2007). Quantitative PCR was performed with an ABI PRISM 7700 equipment using SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. Each 25 µl PCR reaction contained 12.5 µl of qPCR master mix, 5 µl of template from the RT-reaction, and 200 nM forward and reverse primers. PCR cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Measurement of SYBR green fluorescence was performed at the end of each amplification step and continuously during the melt-curve analysis to control for amplicon specificity. The qPCR results and melting curve were analyzed using the SDS 2.1 Software (Applied Biosystems). Control reactions from non-reverse transcribed samples were included to check for residual genomic DNA in the RNA samples. Difference between cycle threshold of reverse transcribed and non reverse transcribed samples was typically higher than 3 cycles indicating that residual DNA accounted for less than 10% in the final result.

Efficiency of PCR amplification was measured using serial dilutions of cDNA from luciferase mRNA or total bacterial RNA after plotting the cycle threshold values versus the log_{10}-transformed number of copies of luciferase mRNA or cells per reaction. Efficiency of PCR amplification was typically in the range of 94 to 99%.
3.3.9 Statistical analyzes

Means of log_{10}-transformed cell counts determined by plate counts and qRT-PCR of heat- and rifampicin tests were compared with Student’s *t*-test using JMP In Software version 6.0 (SAS Institute Inc., Cary, NC, USA) with a level of significance at 0.05.

Table 8 Primers used in qRT-PCR to quantify mRNA fragments of *cysS* and *purB* transcripts

<table>
<thead>
<tr>
<th>Gene or target (locus)</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Amplicon length</th>
<th>Transcript length (location of fragment)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cysS</em> (BL0301)</td>
<td>5’-CAACCGCCCGCAGATCTTC-3’ 5’-CCAGCTGTGAAAGCAACGTATT-3’</td>
<td>57 bp</td>
<td>1641 (1271 – 1327) bp</td>
<td>Rezzonico et al., 2007</td>
</tr>
<tr>
<td><em>purB</em> (BL1800)</td>
<td>5’-CATGGGCGGCGCTTGTG-3’ 5’-TCAAGCTACGCTGACATGAC-3’</td>
<td>57 bp</td>
<td>1446 (254 – 310) bp</td>
<td>Rezzonico et al., 2007</td>
</tr>
<tr>
<td><em>purB</em> (BL1800)</td>
<td>5’-AGCAGGGCCATATCCTTGAA-3’ 5’-TTCTGGCCAACGGCTTTTG-3’</td>
<td>400 bp</td>
<td>1446 (893 – 1292) bp</td>
<td>This study</td>
</tr>
<tr>
<td>Luciferase^a</td>
<td>5’-TACAACACCCCCACACATTCGCA-3’ 5’-GGAAGTTCACCCGCGGTATC-3’</td>
<td>67 bp</td>
<td>1751 (257 – 323) bp</td>
<td>Johnson et al., 2005</td>
</tr>
</tbody>
</table>

^a reference mRNA

3.4 Results

3.4.1 Morphology of *B. longum* NCC2705

Light microscopy of *B. longum* NCC2705 cells from batch cultures exhibited rods in various shapes with the typical V- or Y-pattern. Cells appeared in single cell state and few cell clusters of small numbers were observed (Figure 10 A). Cells originating from continuous cultures with immobilized cells displayed similar shapes but aggregation increased with fermentation time from small aggregates at day 4 (Figure 10 B) to macroscopic aggregates of uncountable cells with a diameter of 0.5 – 1 mm after 9 days of fermentation and remained stable for 20 days of continuous culture (Figure 10 C). Calibration curves for qRT-PCR and stability tests were carried out with free cells from batch cultures.
Figure 10  Optical micrographs of fresh cells collected from fermented broth (1000 × magnification) of *B. longum* NCC2705 produced by batch culture (A), and continuous immobilized cell culture at day 4 (before macroscopic aggregation) (B) and day 16 (after macroscopic aggregation) (C).

### 3.4.2 Calibration curves

Calibration curves of *cysS* 57-, *purB* 57- and *purB* 400-bp mRNA fragments were generated by serially diluting cell samples with concentrations ranging from $7.0 \times 10^3$ to $4.7 \times 10^8$ CFU/ml, corresponding to 9 to $1.7 \times 10^5$ cells per qPCR reaction volume (25 µl). The internal reference of luciferase mRNA was used to account for the variability induced by the RNA extraction procedure with RNA recovery rates ranging between 6 and 70 %.

*C* values for the three amplicons were plotted as a function of the log$_{10}$-transformed CFU/ml determined by plate counting multiplied by the recovery rates of spiked luciferase mRNA (Figure 11). Linear amplification of target mRNAs was measured over a range between 4 and 5.5 log$_{10}$ values, with detection limits of 9 and 20 cells/reaction volumes for short amplicon length of *purB* and *cysS*, respectively, and of 40 cells/reaction volumes for *purB* 400-bp fragments.
Figure 11  Calibration curves obtained by plotting $C_T$ values for cysS 57-bp (A), purB 57-bp (B) and purB 400-bp (C) primer sets as a function of log$_{10}$ transformed cell concentrations determined by plate counts. Data points are from three independent repetitions.
3.4.3 Heat test

Cells were exposed to lethal heat stress and bacterial survival assessed with plate counts was compared with survival obtained with qRT-PCR method. Plate counts showed a reduction of viable cells of $2.7 \pm 0.2$, $3.2 \pm 0.1$ and $3.7 \pm 0.2 \log_{10} \text{CFU/ml}$ after 10, 20 and 30 min exposure at $56 \, ^\circ\mathrm{C}$, respectively (Figure 12). Cell numbers after heat stress determined by qRT-PCR targeting the short amplicons of cysS and purB mRNAs were significantly higher by an average factor of $18.0 \pm 3.3$ and $7.0 \pm 2.0$, respectively, compared to plate counts ($p \leq 0.05$). In contrast, cell numbers measured with qRT-PCR and purB 400-bp fragments were the same as plate counts for all three tested heat stress periods ($p > 0.05$).

![Figure 12](image)

**Figure 12** Viable cell counts of *B. longum* NCC2705 cells measured with qRT-PCR using 3 primer sets and plate counts after heat treatment at $56 \, ^\circ\mathrm{C}$ for 10 (dashed), 20 (grey), 30 min (white) compared to untreated cells (black). Standard deviations of each column are represented by error bars. Different letters indicate significantly different values for each treatment ($p \leq 0.05$).
3.4.4 Rifampicin test

Cells were grown in batch mode until cultures reached OD<sub>600</sub> 0.5 - 0.8 at which point rifampicin was added to stop transcription. The decay of transcripts encoding fragments of cysS and purB of *B. longum* growing at 37 °C was followed at 2.5, 7.5, 14, 20, 25 and 45 min after arrest of transcription (Figure 13).

![Figure 13](image)

**Figure 13** Decay of mRNA fragments of cysS 57-bp (▲), purB 57-bp (■) and purB 400-bp (●) as a function of time after addition of rifampicin at 400 µg/ml to exponentially growing cells. Values are given as corresponding viable cell concentrations derived from calibration curves. Standard deviations of each point are represented by error bars.

The levels of cysS and purB fragments in treated samples were translated into viable cell concentrations using calibration curves. Amplicon levels for cysS 57- and purB 57-bp decreased rapidly within the first 2.5 min after addition of rifampicin (log<sub>10</sub> loss 1.19 ± 0.14, T<sub>1/2</sub> 38.4 ± 4.4 sec) followed by a slower decline until 15 min (1.61 ± 0.25). In comparison, the levels of purB 400-bp amplicons decreased significantly faster (*p* ≤ 0.05) (log<sub>10</sub> loss 1.47 ± 0.03; T<sub>1/2</sub> 30.7 ± 0.7...
sec) and remained at a lower level until 15 min after addition of rifampicin ($\log_{10}$ loss 1.86 ± 0.25). After 15 min, the levels of all three fragments increased steadily. The long fragment of purB reached similar level as its shorter counterpart 20 min after addition of rifampicin.

### 3.4.5 Viable cells quantification of cell aggregates

The qRT-PCR method targeting purB 400-bp fragments was used to quantify viable *B. longum* NCC2705 cells of samples containing macroscopic cell aggregate. Formation of macroscopic cell aggregates (0.5 – 1 mm) was observed after ca. 9 day continuous culture and remained until the end of fermentation (Figure 10 C). Viable cell numbers obtained by measuring purB 400-bp fragment level were compared to plate counts method from samples before and after extensive cell aggregation. Plate counts from samples taken before aggregation (day 4) were lower by 0.5 $\log_{10}$ (approximately 3.2 fold) compared to cell numbers determined with qRT-PCR (Table 8). Samples taken after formation of large aggregates at day 12, 16, and 20 showed larger differences ranging between 2.1, 1.3 and 1.2 $\log_{10}$ units, respectively.

**Table 9** Cell concentrations of *B. longum* NCC2705 during continuous immobilized cell culture determined by plate counts and qRT-PCR (purB 400-bp). Values from day 4 are before formation of macroscopic cell aggregates. Reported values are means of two independent repetitions ± standard deviation.

<table>
<thead>
<tr>
<th>Fermentation day</th>
<th>Cell count (plate count)$^a$ [CFU/ml]</th>
<th>Cell count (qRT-PCR)$^a$ [Cells/ml]</th>
<th>Ratio qRT-PCR/plate counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>4$^a$</td>
<td>3.3 ± 0.3 × 10$^8$</td>
<td>1.1 ± 0.4 × 10$^9$</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>12</td>
<td>6.6 ± 1.9 × 10$^7$</td>
<td>8.6 ± 4.4 × 10$^9$</td>
<td>124.8 ± 26.1</td>
</tr>
<tr>
<td>16</td>
<td>1.8 ± 0.7 × 10$^8$</td>
<td>4.3 ± 2.2 × 10$^9$</td>
<td>22.5 ± 5.7</td>
</tr>
<tr>
<td>20</td>
<td>5.0 ± 2.3 × 10$^8$</td>
<td>7.4 ± 3.3 × 10$^9$</td>
<td>14.7 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ before formation of macroscopic aggregates
3.5 Discussion

Levels of mRNA fragments of housekeeping genes cysS and purB were used for quantification of viable cells of *B. longum* NCC2705 in preparations displaying heterogeneous morphology. These two genes are constitutively expressed in various conditions and used to normalize gene expression analyzes (Rezzonico et al., 2007). The use of unstable mRNA as viability marker is difficult and strongly dependent on the efficiency of RNA extraction because loss during sample preparation significantly biases the output of the experiment (Bustin, 2000). Addition of an exogenous reference mRNA circumvents this bias by monitoring the losses during sample processing. The importance of normalizing mRNA levels with the recovery of exogenous references is highlighted by the large variation in extraction yields ranging from 6 – 70 %. High regression coefficients were obtained for all three mRNA fragments using normalized data with recovery rates. A mean detection limit of $2.7 \pm 1.7 \times 10^4$ cells per ml of culture was achieved for all three mRNA used. This low level is comparable to that reported for bifidobacteria quantified in fecal samples using real-time PCR targeting DNA (Gueimonde et al., 2004). A detection limit of $5 \times 10^4$ cells of *Salmonella* per gram of seeded soil or chicken manure can be achieved using real-time PCR method targeting mRNA extracted with magnetic capture hybridization (Jacobsen and Holben, 2007).

The short mRNA fragments of both housekeeping genes were too stable to be used as accurate viability markers and yielded an overestimation of viable cell counts after the three heat treatments when compared to standard plate counts. In contrast, results obtained with long fragments of *purB* coincided well with plate counts irrespective the duration of the stress treatment. Differential stability of specific mRNA fragments was confirmed by treating *B. longum* NCC2705 with rifampicin, an inhibitor of RNA-transcription (Campbell et al., 2001).
The 400-bp fragments of *purB* were degraded faster than short fragments of both genes. Similar inverse relation between length of RNA or DNA molecules and their stability was recently reported in other bacterial species (Aellen *et al.*, 2006, Soejima *et al.*, 2008). Quantification of rRNA levels after antibiotic treatment in *Streptoccus gordonii* showed better correlation between qRT-PCR values and plate counts when targeting a 427-bp compared to a 119-bp fragment which remained stable after drug killing (Aellen *et al.*, 2006). DNA was used as a viability marker of *Listeria monocytogenes* in combination with a DNase-assay and showed that long DNA fragments (894-bp) of heat killed cells were not detectable after treatment, whereas short fragments (113-bp) were still evident causing false positive results (Soejima *et al.*, 2008).

The rapid decrease of levels of the three fragments in the first minutes after addition of rifampicin yield very small half-life times (33.6 ± 3.4 sec) compared to that reported for *Escherichia coli*, *Bacillus subtilis* and *Lactococcus lactis* of 3-8 min (Bernstein *et al.*, 2002; Hambraeus *et al.*, 2003; Redon *et al.*, 2005). However, mRNA half-lives in these studies were calculated from the degradation rate constant obtained from microarray analyzes. The use of sensitive qRT-PCR technique can affect the determination of RNA levels compared to microarray results. Decay rates of transcripts determined with microarray technology were shown to be slower than values obtained by Northern blots or real time PCR (Hambraeus *et al.*, 2003; Hundt *et al.*, 2007).

The increase of transcript levels shortly after the start of the treatment can be attributed to rapid bacterial adaptation to rifampicin or selection for resistant subcultures within the same culture as reported for bifidobacteria from human samples treated with this antibiotic (Mangin *et al.*, 1994). Proliferating cells regenerate new intact mRNA transcripts resulting in equal levels of *de novo* synthesized transcripts of short and long *purB* fragments as observed 20 min after beginning of treatment.
Although auto-aggregation might improve adhesion of probiotic bacteria to epithelial cells (Del Re et al., 2000), application of aggregated cells in functional food products suffers from underestimation of viable cell counts obtained with standard plate count since the method cannot detect individual cells in aggregates. The use of cell aggregates is thus still unfavorable in product applications with plate counts being the standard method to verify product quality. In fact, the use of the qRT-PCR method with aggregated cells showed that plate counts largely underestimated viable cell numbers with factors ranging from 15 to 125.

In conclusion, we developed an accurate real time PCR method determining viable cell counts of _B. longum_ NCC2705 with different morphologies. The careful selection of the target gene and fragments length as viability markers is essential to avoid biases in viable cell counting. Comparison of primer sequences of _purB_ 400-bp with sequenced genomes showed direct hits with genomes of _B. longum_ DJO10A and _B. longum_ subsp. _infantis_ ATCC 55813, CCUG 52486 and ATCC 15697, an auto-aggregating strain (Rahman et al., 2008) and could therefore be used as viability marker in these strains. qRT-PCR may be used for rapid quantification of viable cells in mixed cultures provided that primer specificity and sample processing is not affected by mixed culture conditions. Moreover, the method may be developed in combination with DNA quantification to evaluate the proportions of dead cells within cell aggregates which may provide a protection layer for viable cells inside the core of aggregates during transit through the GIT.

Growing interest in probiotic characteristics of aggregating bacterial cells make this technique a valuable tool to accurately quantify viable probiotic bacteria exhibiting heterogeneous morphology.
4 Development of a rapid screening protocol for selection of *Bifidobacterium longum* strains resistant to spray drying and powder storage

Data presented in this chapter were submitted and accepted with minor revision in 2009 in Beneficial Microbes with the following authors: Reimann S., Grattepanche F., Baggenstos C., Rezzonico E., Berger B., and Lacroix C.
4.1 Abstract

An efficient screening method for selection of *Bifidobacterium longum* strains resistant to spray drying and storage was developed based on Randomly Amplified Polymorphic DNA (RAPD) for identification of the best survivors in mixed strains bacterial preparations. Three different primers were used to generate RAPD profiles of 22 *B. longum* strains. All strains were distinguished according to their RAPD profiles except for the strain NCC2705 and its H$_2$O$_2$ resistant derivative variant. The 22 strains were grouped in 3 batches of 7, 7 and 8 strains and subjected to spray drying and storage at 30 and 37 °C under anaerobic conditions. Batch survival rates after spray drying reached 17.1 ± 4.4 %. Strains showing the highest prevalence and/or resistance to storage at 37 °C were selected from individual batches for subsequent spray drying and storage testing. After 67 days of storage, NCC572 was identified as dominant strain in powder. The stability of strain NCC572 was confirmed by performing single spray drying and storage tests. Out of 22 *B. longum* strains, a robust strain was identified by combining RAPD with a simultaneous screening test for survival to spray drying and storage. The method allowed a fast screening of *B. longum* strains in mixture for resistance to spray drying and storage compared to traditional screening procedures carried out with individual strains, in the same conditions. This approach could be applied to other stress conditions.
4.2 Introduction

Bifidobacteria are widely used as probiotics in functional food products. Industrial standards currently used for products containing viable probiotic strains require presence of minimal bacterial viable counts in order to confer health benefits to consumers. Probiotic bacteria in functional food products are usually incorporated as frozen concentrates or freeze dried powders (Meng et al., 2008; Saarela et al., 2006). Freezing and freeze drying are however expensive stabilisation technologies due to the infrastructure and energy consumption during production, transport and storage (Knorr, 1998). Spray drying has gained popularity over the last decade for large scale production of starter cultures and probiotics (Corcoran et al., 2004; Desmond et al., 2002; Gardiner et al., 2000; Lian et al., 2002; Meng et al., 2008; O’Riordan et al., 2001; Simpson et al., 2005). Although spray drying is an economical process, between 5 – 10 times cheaper than freeze drying (Santivarangkna et al., 2007), bacterial cells suffer lethal damages attributed to the effect of exposure to heat and dehydration (Ross et al., 2005; Teixeira et al., 1995a). These have deleterious effects on survival of sensitive bacteria such as probiotics. Survival rates of probiotic bacteria during spray drying is strain-dependent and influenced by several factors, the most important being the drying conditions applied, in particular outlet temperature (Gardiner et al., 2000; Lian et al., 2002), the carrier media used (Corcoran et al., 2004; Johnson and Etzel, 1993; Lian et al., 2002;), presence of protective compounds and the growth phase of cells (Meng et al., 2008).

Loss of bacterial viability during storage of dry cultures is also a major limiting factor for many cultures (Ross et al., 2005; Teixeira et al., 1995). Parameters such as temperature, moisture content of spray dried powders, water activity, carrier media, exposure to light and oxygen have been shown to impact bacterial survival during storage in powder form (Ananta et al., 2005;
Chavez and Ledeboer, 2007; Gardiner et al., 2000; Meng et al., 2008; Simpson et al., 2005; Teixeira et al., 1996). Spray drying and storage survival rates do not concur and vary from strain to strain (Corcoran et al., 2004; Gardiner et al., 2000; Simpson et al., 2005) and therefore careful strain selection is important to ensure high survival during spray drying and subsequent storage of dried cultures. The underlying molecular mechanisms responsible for viable cell losses during drying and storage remain unclear and thus empirical approaches for efficient strain screening are of utmost value to develop probiotic strains with good technological and functional properties and to avoid labour-intensive, lengthy and costly strain-by-strain screening procedures.

Molecular typing techniques such as restriction fragment length polymorphism, pulse field gel electrophoresis, repetitive element sequence-based PCR fingerprinting and randomly amplified polymorphic DNA (RAPD) allow the differentiation of bacteria at strain level without prior knowledge of genome sequence (Ben Amor et al., 2007; Collado et al., 2006; Gevers et al., 2001; Masco et al., 2003; Rossetti and Giraffa, 2005; Vincent et al., 1998).

In this study, we applied an efficient method using RAPD-based strain identification to identify the best survivor strains after spray drying and storage of mixed bacterial preparations during two selection rounds followed by a final confirmation step (Figure 14). Twenty two *B. longum* were divided into three batches of 7 or 8 strains and exposed to a first selection consisting of a spray drying and storage experiment. Best survivors of each batch preparation were identified by RAPD after 4 log₁₀ viability loss during storage and applied to a second selection round to identify the most resistant *B. longum* strain. The survival of the selected strain was tested separately and compared to a low resistant strain for validation.
Figure 14 Flow chart of experimental procedure. The 22 \textit{B. longum} strains were divided into 3 batches for spray drying and storage. After the first selection round, 2 - 3 best survivor strains were selected for a subsequent spray drying and storage batch. After the second selection round, the best survivor strain and a control strain were selected for a single strain spray drying and storage confirmation batch.

4.3 Material and Methods

4.3.1 Bacterial strains and culture conditions

The 22 \textit{B. longum} strains used in this study were provided by Nestlé Culture Collection (NRC, Lausanne, Switzerland) (Table 10). Strains were routinely cultured in 20 ml-McCartney bottles containing de Man, Rogosa, Sharpe (MRS) broth (BioLife, Milan, Italy) supplemented with 0.05 \% (w/v) L-cysteine (Sigma-Aldrich, Buchs, Switzerland) (MRSC), and incubated for 16 h at 37
°C in anaerobic conditions using atmosphere generation system packs (AnaeroGen, Oxoid, Pratteln, Switzerland). Cultures from frozen stock were sub-cultured twice before use as inoculum at 1% (v/v). Cell counts of pure liquid cultures were determined by serial dilutions of samples in 12 mM phosphate buffer saline with 0.05 % (w/v) L-cysteine (PBSC) followed by plating in duplicate on MRSC agar 1.5% (w/v) (Becton Dickinson, Basel, Switzerland). Plates were incubated anaerobically at 37 °C for 72 hours.

4.3.2 Preparation of bacterial suspensions used for survival tests

Individual strains were grown separately at 37 °C in rubber-lid flasks containing 250 ml MRSC inoculated at 2 % (v/v). The headspace of flasks was flushed with CO$_2$ to ensure anaerobic conditions. Growth was monitored by measuring OD$_{600}$ and cells were harvested 3 h after reaching stationary growth phase. Volumes of 40 ml of cell broth were centrifuged (15,000 × g, 15 min at 4 °C), and cell pellets washed once with PBS-C and resuspended in 100 ml ice-cold 20 % (w/v) reconstituted skim milk (RSM) (Oxoid Ltd, Hampshire, United Kingdom). A sample of 1 ml was used for plate counts.

4.3.3 Preparation of strain mix

For the first and second selection batches, 100 ml of each bacterial suspension (7 or 8 strains per batch) in RSM were mixed together in a 1 l-bottle containing a magnetic stirrer (Table 10). The solution was kept at 4 °C in an ice bath and stirred for at least one hour before spray drying. For single strain confirmation tests, a similar procedure was used with pure cultures to produce 400 ml ice-cold RSM cell suspension used for spray drying.
4.3.4 Spray drying

Spray drying was conducted using a laboratory scale spray dryer (Büchi model B-290, Büchi, Flawil, Switzerland). The feed suspension was pneumatically atomized into a vertical, co-current drying chamber using a two-fluid nozzle. The inlet air temperature was maintained at 170 °C and the outlet air temperature was adjusted to 78.2 ± 1.9 °C by levelling the feed pump rate. A maximum volume of 300 ml of bacterial RSM mix was used per batch corresponding to a maximum of 3 h operation time. The spray dried powder was collected in a single cyclone separator.

Spray drying was performed once for the first selection batches whereas second selection batch and confirmation experiments using single strain preparations were carried out in duplicate.

4.3.5 Storage test

Spray dried powder was aliquoted in 2-ml polypropylene tubes (Eppendorf, Hamburg, Germany) sealed with sterile cotton wool. Tubes were then placed in 2.7 l desiccators (DURAN Group GmbH, Mainz, Germany) containing 150 ml of 5 M saturated potassium acetate as desiccant and kept in the dark. Storage temperatures of 30 and 37 °C were tested for first selection batches whereas powders from second selection and confirmation batches were stored at 37 °C only. Desiccators were evacuated and then flushed with nitrogen to ensure anaerobic conditions. Powders from selection batches were sampled 2-3 times per week or once a week towards the end of storage or in confirmation batches for cell counts and water activity measurement. Storage was stopped when a threshold loss in cell counts of ca. 4 log_{10} of number of colony forming units (CFU) per gram of powder was reached. Storage experiments were performed once per spray
drying batch for the first selection batches and in duplicate per spray drying experiment for the second selection batches.

4.3.6 Determination of water activity and moisture content

Water activity of powder was measured using a hygrometer (AquaLab, Gerber Instruments SA, Effretikon, Switzerland) according to the manufacturer’s recommendations. Values are means of two determinations per sample taken at a reference temperature of 25 °C.

Moisture content expressed as percentage of spray dried powder weight was determined by measuring the difference of powder weight before and after heating 10 g of powder overnight at 102 °C. Dry matter of bacterial suspension in RSM was determined by weighing 10 ml of suspension oven-dried for 36 hours at 80 °C. Determination of moisture content and dry matter was performed in duplicate.

4.3.7 Viable cell counts in spray dried powders

Survival of B. longum strains to spray drying and storage was performed according to Gardiner et al. (2000). Briefly, 0.1 g powder was resuspended in 9.9 ml PBS-C, homogenized using a stomacher 80 (Müller and Krempel AG, Bülach, Switzerland) for 30s and stored at room temperature for 1 hour to allow rehydration. Cell suspensions were serially diluted in PBSC and appropriate dilutions were plated in duplicate on MRS-C agar. Percentage of survival after spray drying was calculated as the CFU per gram powder after spray drying divided by the initial number of CFU per gram dry matter in RSM bacterial suspensions before spray drying.
4.3.8 Identification of best surviving strains using RAPD

Genomic DNA was extracted from pure cultures grown in liquid media according to Goldenberger et al. (1997). Briefly, 0.5 ml of culture were centrifuged and resuspended in 0.2 ml digestion buffer containing 50 mM Tris-base (Sigma-Aldrich), 1 mM EDTA (pH 8.5) (Sigma-Aldrich), 0.5 % SDS (Sigma-Aldrich), 200 µg/ml proteinase K (AppliChem GmbH, Darmstadt, Germany). After incubation at 55 °C for 3 h under agitation (Thermomixer, Eppendorf, Hamburg, Germany), the mixture was held at 95 °C for 10 minutes to inactivate proteinase K, cooled to 4°C and centrifuged (12,000 × g, 10 min, at 4 °C). The supernatant was collected and nucleic acid concentration was determined using Nanodrop ND-1000 (Peqlab, Erlangen, Germany). Samples were diluted in distilled water to reach a final nucleic acid concentration of 25 ng/µl and stored at – 20 °C until PCR amplification.

RAPD analysis was carried out according to Vincent et al. (1998), i.e., one µl of DNA sample (25 ng/µl) was mixed with 24 µl mastermix containing 0.2 mM dNTP (GE Healthcare Europe, Glatttbrugg, Switzerland), 0.2 pmol of each primer, 10 x Taq buffer (Euroclone, Siziano, Italy), 1.5 mM MgCl$_2$ (Sigma-Aldrich), 2 % (w/v) Tween 20 (Sigma-Aldrich) and 2.5 U Taq Polymerase (Sysmex Digitana, Horgen, Switzerland). Three random single primers were used: OPA-02 (5’ TGCCGAGCTG 3’), OPL-07 (5’ AGGCGGGAAC 3’ ) and OPL-16 (5’ GGGAACGTGT 3’) (Microsynth, Balgach, Switzerland). These three primers were selected because they permit differentiation of all 22 strains except for NCC2705 and its H$_2$O$_2$-resistant variant NCC2916 (data not shown) which could be differentiated according to physiological H$_2$O$_2$ resistance tests (Figure S1). These two strains were distributed in two different batches during the first selection batch experiments to avoid identification problems (Table 1). PCR amplification was run in a Biometra®TGradient thermocycler (BioLabo, Châtel-St. Denis,
Switzerland) under the following cycling conditions: 94 °C for 3 min, 45 cycles of 94 °C for 1 min, 30 °C for 1 min and 72 °C for 2 min. PCR products were run on 2 % (w/v) agarose gel stained with ethidium bromide. The RAPD profiles were recorded using Alphalmager™ system (Alpha Innotech Corporation, California, USA).

Survival of individual strains after spray drying and storage of dry powder containing mixed strains was measured after reaching 4 log₁₀ loss in total bifidobacterial counts. A total of at least hundred colonies from 2 - 4 plates harboring between 5 and 100 colonies were then randomly picked and separately cultivated in MRSC broth at 37 °C for 24 h under anaerobic conditions and stored at -20 °C until DNA extraction.

4.4 Results

4.4.1 Strain survival and selection during the first selection batches

22 \textit{B. longum} strains were divided in three batches each consisting of 7 - 8 strains (Figure 14) for spray drying and storage experiments (Table 10). Mean initial cell concentration of cell suspensions in RSM for the three batches before spray drying was $1.5 \pm 0.4 \times 10^9$ CFU/g dry matter. Viable total cell counts of spray dried powders measured immediately after spray drying were 2.2, 3.6 and $1.8 \times 10^8$ CFU per gram of powder, corresponding to survival rates of 12.6, 21.4 and 17.4 % for Batch 1, 2 and 3, respectively. Average moisture content of powders was 5.4 ± 0.7 %. Aliquots of dried powder were then stored in two desiccators at 30 and 37 °C with a water activity of 0.23 ± 0.01.
Table 10  Distribution of the 22 *B. longum* strains in the three batches of the first selection round and origin of the strains.

<table>
<thead>
<tr>
<th>Strain(^a)</th>
<th>Batch number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCC284</td>
<td>1</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC293</td>
<td>1</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 305</td>
<td>1</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 324</td>
<td>1</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 344</td>
<td>1</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 2705</td>
<td>1</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 3001</td>
<td>1</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 417 (ATCC 15707)</td>
<td>2</td>
<td>Fermented milk (yogurt)</td>
</tr>
<tr>
<td>NCC 435 (ATCC 15708)</td>
<td>2</td>
<td>Adult feces</td>
</tr>
<tr>
<td>NCC 444 (NCIMB 8809)</td>
<td>2</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 450 (DSM 20097)</td>
<td>2</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 461</td>
<td>2</td>
<td>Calf feces</td>
</tr>
<tr>
<td>NCC 469</td>
<td>2</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 4012</td>
<td>2</td>
<td>Commercial probiotic product</td>
</tr>
<tr>
<td>NCC 490</td>
<td>3</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 510</td>
<td>3</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 521</td>
<td>3</td>
<td>Adult feces</td>
</tr>
<tr>
<td>NCC 552</td>
<td>3</td>
<td>Adult feces</td>
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<tr>
<td>NCC 572</td>
<td>3</td>
<td>Infant feces</td>
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<tr>
<td>NCC 585</td>
<td>3</td>
<td>Adult feces</td>
</tr>
<tr>
<td>NCC224</td>
<td>3</td>
<td>Infant feces</td>
</tr>
<tr>
<td>NCC 2916</td>
<td>3</td>
<td>H(_2)O(_2) resistant mutant of NCC2705</td>
</tr>
</tbody>
</table>

\(^a\)Alternative strain denotation indicated in brackets.  
ATCC: American Type Culture Collection; DSM: Deutsche Sammlung von Mikroorganismen; NCIMB: National Collection of Industrial, Marine and Food Bacteria; NCC: Nestlé culture collection

A non-linear decrease of cell counts (log\(_{10}\)) was observed in spray dried powders during storage at both temperatures (Figure 2). Powder storage at 37 °C resulted in faster viability loss with a 4 log\(_{10}\) loss threshold in cell counts reached after 46, 40 and 49 days at 37 °C (Figure 2 B) compared to 64, 65 and 53 days at 30 °C (Figure 2 A) for Batch 1, 2 and 3, respectively. After this storage period, at least 100 individual colonies isolated from different plates of the highest plating dilutions were collected and identified using RAPD.
Figure 15   Cell counts in spray dried powders of batches from first selection round during storage at 30 °C (A) and 37 °C (B) for Batch 1 (full line, ●), 2 (semi-dashed line, ▲) and 3 (dashed line, ■).
Proportion of individual strains found within the three batches before and after spray drying and storage at 37 °C are shown in Figure 16. Different fractions were already measured for individual strains before spray drying as a result of different growth of cells in the culture used for preparation of the bacterial strain mixtures in RSM. After spray drying and storage of Batch 1 at 37 °C, all seven strains present in the initial cell suspensions were detected at the end of the storage period. NCC3001 and NCC293, which were predominant before spray drying showed only a small decrease of their relative proportion after storage, from 32 and 45 % to 30 and 40 %, respectively (Figure 16 A). NCC284 was the only strain showing increased relative proportion after storage (from 4 to 10 %). Only three of the seven strains present in the initial cell suspension were detected after storage at 30 °C. The relative percentage of strain NCC3001 increased from 32 to 56 % whereas NCC293 remained stable at about 44 %. NCC284 was not detected at the end of storage at 30 °C (Figure 16 A).

Similarly to Batch 1, strain diversity in Batch 2 and 3 was also better conserved after storage at 37 °C compared to 30 °C. In Batch 2, strains NCC417 and NCC435 were already over-represented before spray drying and displayed the highest stability since their relative proportion increased after storage at both 30 and 37 °C, from 29 to 36 and 50 % for NCC417 and from 23 to 30 and 43 % for NCC435, respectively (Figure 16 B). Surprisingly, NCC461 doubled its relative presence from 13 to 29 % in the powder stored at 30 °C, but its fraction decreased to only 1 % at 37 °C. In Batch 3, strain NCC572 initially accounting for 22 % of total bacterial counts, largely dominated after storage, reaching 95 and 98 % at 30 and 37 °C, respectively (Figure 16 C).
Figure 16    Relative presence (%) and corresponding cell counts (CFU per gram) of *B. longum* strains in RSM before spray drying and in spray dried powders after end of storage at 30 and 37 °C for the first selection Batches 1 (A), 2 (B) and 3 (C). Storage was stopped after 4 log10 loss of CFU counts in powder (in brackets: storage time). For RSM, distribution-profiles were calculated using cell concentration of each strain in pure culture before mixing. Individual populations in spray dried powders were estimated using their relative abundance (%) determined by RAPD and total cell counts in powder.
In order to reduce the number of strains for the second selection round, only 2 or 3 strains from each batch were selected based on their high survival in powders stored at 37 °C. Strains with highest percentage (NCC293) or increased relative percentage (NCC 284) were selected among the 7 strains detected in the powder for Batch 1 (Figure 16 A). For Batch 2, among the 5 strains detected in the powder, strains NCC417 and NCC435 were selected due to their high fraction in the RSM suspension (indicating good growth characteristics in MRSC) and their increased relative proportion after storage. NCC450 was also selected because its fraction in the RSM cell suspension and in the powder after storage remained stable (Figure 16 B). For Batch 3, among 3 strains detected in the powder after storage at 37 °C strains NCC572 and NCC585 were chosen due to their dominant appearance (NCC572) and stable percentage (NCC585) (Figure 16 C). Strain NCC2916 which showed low survival in Batch 3, was chosen as a “negative control” for inclusion in the second selection round.

### 4.4.2 Strain survival and selection during the second selection round

Selected strains were spray dried and stored at 37 °C. The moisture content and water activity in spray dried powders from the second selection round with 8 strains were 5.4 ± 0.2 % and 0.21 ± 0.01, respectively. Mean initial cell concentration before spray drying in RSM was 1.2 ± 0.2 × 10^9 CFU/g dry matters. Survival during spray drying was 16.2 ± 9.1 % and cell counts in the powder before storage were 2.0 ± 1.3 × 10^8 CFU/g. The log_{10} transformed cell counts in spray dried powder decreased non-linearly with storage time (Figure 17), but at a slower rate compared to the first selection batches at 37 °C (Figure 2 B). Therefore, cell counts did not reach the 4 log_{10} loss threshold per gram of powder after 67 days of storage (Figure 17).
**Figure 17**  Cell counts in spray dried powders of second selection batch (8 strain mix) (full line, ○) and confirmation batches NCC572 (semi-dashed line, ■) and NCC2916 (dashed line, ▲) during storage at 37 °C. Data are means of two independent experiments. Standard deviations of each point are represented by error bars.

Individual strains accounted for 6 to 20 % of total cell counts in the RSM mix (Figure 18 A). RAPD identification of 239 colonies picked after 67 days storage at 37 °C indicated strain NCC572 as best survivor after spray drying and storage, with a large proportional increase in powder from 12 % in RSM before spray drying to 88 % in the powder (Figure 18 B). NCC585 also exhibited good survival in powder with constant proportion of total cells of about 9 ± 2 % in the RSM mix and powder. All the other strains decreased in percentage or were not detected in the powder (Figure 18 B).
Figure 18  Relative presence (%) and corresponding cell counts (CFU per gram) of the *B. longum* strains of the second selection batch in RSM before spray drying (A) and in spray dried powders after storage for 67 days at 37 °C (B). For RSM, distribution-profiles were calculated using cell concentration of each strain in pure culture before mixing. After storage, individual populations in spray dried powders were estimated using their relative abundance (%) determined by RAPD and total cell counts. Data are means of two trials ± standard deviation.

\(^a\) No standard deviation since only one colony was picked.

### 4.4.3 Validation of selection procedures using single strain preparations

To confirm NCC572 as good spray drying and storage survivor, NCC572 and NCC2916 (low survivor control) were tested in pure culture spray drying and storage experiments. Average moisture content and water activity of powders was 5.5 ± 0.6 % and 0.17 ± 0.01, respectively. Survival rate of NCC572 and NCC2916 during spray drying was 18.8 ± 9.6 and 4.8 ± 3.9 % with an initial viable cell count of $2.3 \pm 2.7 \times 10^9$ and $9.7 \pm 2.0 \times 10^8$ CFU/g dry matter, respectively. Strain NCC2916 showed a 4 log\(_{10}\) loss threshold in cell counts after 32 days of storage whereas
strain NCC572 exhibited only $2 \log_{10}$ viability loss after 34 days and $3.4 \log_{10}$ after 69 days (Figure 17). Viable cell counts in powders of NCC572 after 62 days of storage were $1.5 \pm 0.5 \times 10^5$ CFU/g powder and exceeded counts of NCC2916 after 60 days by a factor of 269.

4.5 Discussion

Spray drying and storage stability is an important bacterial characteristic for the use in food application at industrial scale because spray drying is a low cost alternative to freeze drying. Survival to drying and storage is strain dependent and selection of robust strains with good functional properties is required for successful application. Screening of optimum process conditions and strains with good resistance is laborious, expensive and limited by variability between experiments. Simultaneous screening of multiple strains can be used to increase screening throughput. RAPD is a fast, practical, easy to perform and inexpensive technique to distinguish bacterial strains. Furthermore, its application does not require a high level of technical skills (Rossetti and Giraffa, 2005). RAPD analysis was successfully used in this study to discriminate 22 *B. longum* strains, except strain NCC2705 and its H$_2$O$_2$ resistant derivative NCC2916, in mixtures of up to 8 strains. It is theoretically possible to apply all 22 strains in one single spray drying and storage batch provided that uniform treatment for each strain is guaranteed.

Spray drying parameters were chosen from previous studies with bifidobacteria and lactic acid bacteria and kept constant for all batches. RSM as a carrier medium is commonly used for spray drying of lactic acid bacteria due to its protective effect against heat (Ananta et al., 2005; Desmond et al., 2002). The inlet and outlet temperatures were set at 170 °C and ca. 80 °C, respectively, according to Gardiner et al., (2000). Survival rate of *B. longum* cells determined
after spray drying in mix or pure cultures ranged from 5 to 21 % under these conditions and concurred well with survival rate of 20 ± 1 % reported for B. longum biotype longum NCIMB 8809 in 20 % (w/v) RSM solution (Simpson et al., 2005), a strain also used in this study (NCC444). The different survival rates between batches are most likely caused by different strain compositions. Strain dependent survival to spray drying was previously reported for bifidobacteria ranging between 12 – 102 % with an outlet temperature of 85 – 90 °C (Simpson et al., 2005). Conditions were set to achieve a moisture content of the powder close to the optimal value of 4 % for stability during storage (Gardiner et al., 2000; Simpson et al., 2005). However, a slightly higher moisture value of 5.4 ± 0.7 % of powders was obtained in our study, partly explained by a lower outlet temperature of 78 °C. Increasing the outlet temperature to produce a cell powder with 4 % moisture content would likely decrease survival rate after spray drying as reported by Gardiner et al. (2000) and Lian et al. (2002) for spray drying of Lactobacillus paracasei, Lactobacillus salivarius and bifidobacteria. Water activity of 0.23 ± 0.01 during storage for all batches containing strain-mixes lies within the recommended range for stability of dried lactic acid bacteria (Champagne et al., 1996; Teixeira et al., 1995b).

The log$_{10}$ transformed cell counts in powder decreased non-linearly as shown for lactobacilli stored in RSM powder at 30 °C (Gardiner et al., 2000). Storage temperatures of 30 and 37 °C selected to accelerate the loss in cell viability caused a faster decrease in cell viability at 37 than at 30 °C and reached a 4 log$_{10}$ units loss within 5 - 10 weeks. This effect of temperature is in agreement with other studies on survival of spray dried probiotic bacteria during storage (Corcoran et al., 2004; Desmond et al., 2002; Gardiner et al., 2000; Simpson et al., 2005; Teixeira et al., 1995b).

Survival of the single strains varied among powders stored at 30 and 37 °C and resulted in differences of strain distribution in the two powders. Remarkably, a higher number of different
strains was detected after storage at 37 °C compared to 30 °C for Batch 1 and 2, and to lesser extent for Batch 3. The time period of storage may have a noticeable impact on survival of each strain in dried powders because time duration between collection of samples at 30 and 37 °C differed between 18 and 25 days for Batch 1 and 2, respectively compared to Batch 3 where samples from storage at 30 °C where taken after only 53 days of storage, 4 days after the sampling from storage at 37 °C. These findings suggest that longer storage periods at lower temperatures may raise selection pressure to strains in powder state leading to a lower variety of strains detectable at the end of storage at 30 °C.

The second selection round aimed at identifying the best survivors among strains selected from the first round. The assorted strains in the second selection batch exhibited as expected prolonged survival in powder and did not reach the $4 \log_{10}$ loss threshold after 67 days of storage compared to first selection batches where thresholds were reached within less than 50 days. Survival to spray drying and storage for 67 days at 37 °C varied largely among single strains and led to identification of strain NCC572 as best survivor strain among 22 tested $B.\ longum$ strains. Its high survival was confirmed by single strain drying and storage tests.

Successful application of spray drying of probiotic strains as an economic alternative to freezing or freeze drying depends on the careful selection of appropriate strains harbouring intrinsic resistance to drying and powder storage in addition to aspects of safety and functionality. Our study showed the potential of RAPD technology to screen strains for survival to drying and storage in combination with a time-effective mixed strain screening approach. A similar method combining strain mixture testing and RAPD detection could be used to screen probiotic strains simultaneously for resistance to different lethal stresses related to technological and functional properties such as gastric, bile, oxygen, low pH, heat, and osmotic stress. Moreover, this approach allows direct comparison of stress response of single strains and avoids variation.
between experiments and time consuming repetitions. Further analyses of the strains exhibiting intrinsic tolerance to drying and storage using comparative genomics and transcriptomics tools could be followed in order to try to elucidate mechanisms conferring microbial stability during drying and storage.
5 General conclusions and outlook

5.1 General conclusions

The PhD thesis “Novel technologies for detection, production and screening of stress tolerant bifidobacteria” consisted of three hypotheses. The first hypothesis claimed that \( B.\ longum \) NCC2705 produced with immobilized cell technology and continuous culture had an improved stress resistance progressing with culture age. In previous studies, cell immobilization in combination with continuous probiotic culture showed positive effects on environmental stress tolerance improving with culture time. Our studies confirmed the hypothesis partly, since we observed better tolerance of immobilized cells (IC) of \( B.\ longum \) NCC2705 to porcine bile salts and some antibiotics compared to free cells from batch culture independent of the culture age, whereas tolerance to heat stress did not change, neither with culture age nor compared to batch control cells. However, a time dependent change of cell morphology was detected and resulted in formation of macroscopic cell aggregates of \( B.\ longum \) NCC2705. This extensive morphological changes lead to great difficulties in accurately determining viable cell numbers which are prerequisite for testing cell stress tolerance. Continuous immobilized cell fermentation in a two-stage process lead to high cell production, similar to bacterial batch cultures and increased cell volumetric productivity by approximately ten fold.

The immobilization of \( B.\ longum \) NCC2705 did not concur with the observations of Doleyres et al. (2004a) where immobilized cells of \( B.\ longum \) ATCC 15707 produced continuously in coculture with \( Lc.\ lactis \) showed increased tolerance to various environmental stresses and no formation of cell aggregates. The differences in stress resistance as well as the induction of autoaggregation may be attributed to the strain specificity in addition to difficult viable cell determination. Moreover, the enhanced robustness of \( B.\ longum \) ATCC 15707 may be a result of
growth in the immobilized matrix in combination with an adaptation process to co-culture conditions rather than only from cell immobilization. In fact, a recent study showed that only the supernatant of co-culture in a compartmentalized fermentation with a \textit{B. longum} and \textit{B. breve} strain affected bacterial physiology (Ruiz et al., 2009). Improved tolerance to porcine bile salts and auto-aggregation of \textit{B. longum} NCC2705 produced in our study could confer advantages in the probiotic approach. Large cell aggregates might provide physical protection from adverse environmental parameters during transit through GIT and could improve adhesion abilities to mucosal epithelium from the host. Although the acquired characteristics could be partly explained by the observed changes in the fatty acid composition, other factors may also contribute to altered cell physiology. Preliminary results from microarray analyzes showing transcriptional regulation of membrane associated transporter proteins as well as ribosomal proteins may reflect a deep impact of the culture conditions on transport mechanisms and translation machinery (see Appendix 6.2).

The acquired physiological and morphological characteristics of cells from ICC should be tested for reversibility by repeated batch cultures and the predicted advantage in adhesion capacity could be testing using \textit{in vitro} assays with intestinal cells such as Caco-2 by testing large cell aggregates versus batch control cells. However, physiological analyzes of bacteria in large aggregates are impossible to perform with traditional plate count method.

In the second part of the thesis, we developed thus a new protocol to measure viable \textit{B. longum} NCC2705 cells using real time PCR technology. The method was based on measurement of transcript levels of a housekeeping gene using quantitative real time PCR (qRT-PCR). The development of the protocol is based on the detection of mRNA of a housekeeping gene whose constitutive expression was confirmed with microarray analyzes (data from Nestlé Research Center Lausanne, Switzerland). The hypothesis was verified in that an adequate viability marker
was identified as an amplicon of 400 bp length of purB gene of \textit{B. longum} NCC2705. Batch free cells quantified with qRT-PCR showed good correlation with plate counts before and after a lethal heat stress treatment. The quantification of cells in the aggregated state with qRT-PCR confirmed that the traditional plate count method resulted in major underestimation of viable cells counts.

Quantification of viable cells in macroscopic aggregates revealed the limitation of the traditional plate count method and emphasized the need of new technologies to quantify viable bacteria. The qRT-PCR method developed in this method is not limited to quantify viable \textit{B. longum} NCC2705 cells in pure cultures only. The method could be applied to other bifidobacterial strains or used to quantify \textit{Bifidobacterium} subsp. in mixed cultures with bacteria belonging to different genera, provided that enzyme activity and primer specificity remains unaffected. Drawbacks of the method is the requirement of well-trained labor as well as the high cost of enzymes, fluorescent stains and RNA-extraction which limit the extensive application of the method as a standard protocol. Progressive use of such consumables for gene expression analyzes such as RT-PCR or microarray analyzes might lower the cost in the near future and therefore paves the way for broad application. Beyond the quantification of probiotic bacteria, the method could be a valuable tool to examine the viability of pathogenic microorganism in biofilms. The growing number of sequenced microorganisms will increase the knowledge of gene expression patterns and broaden the applicability of rapid and accurate molecular quantification tools which will lead to sophisticated quantification of viable bacteria independent of the morphological state.

In the last part of the thesis, we used a different approach to identify stress tolerant \textit{B. longum} strains. We aimed at developing an efficient strategy to screen a number of \textit{B. longum} strains with intrinsic resistance to conditions of spray drying and accelerated powder storage. We hypothesized the possibility to apply a number of \textit{B. longum} strains to spray drying and storage
experiment simultaneously and identify the best survivor strain(s) using random amplified polymorphic DNA (RAPD) method at the end of the storage test. Twenty-two strains of bifidobacteria belonging to *B. longum* species could be distinguished using this approach that allowed identifying one strain with enhanced survival rate to drying and storage. This higher resistance was confirmed by single strain spray drying and storage experiment and by comparing the results with a poor survivor strain.

The rapid screening protocol developed for the spray drying and storage approach can be projected to various other stress tests relevant for production and consumption of probiotic food products. An additional empirical approach with the same 22 *B. longum* strains applied to freeze drying and subsequent accelerated storage might deliver important insights about parallels and differences of survival of each strain to spray- and freeze-drying followed by storage.

### 5.2 Outlook and perspectives

The technologies and protocols applied in the thesis can be also applied alone or combined in new approaches to produce or select stress tolerant probiotic strains. Continuous culture with immobilized cells for instance can be applied to other probiotic strains to test whether induction of aggregation and bile tolerance is strain dependent or not. The high throughput screening protocol could be used to monitor survival to simulated gastrointestinal conditions or even *in vivo*, with humans or gnotobiotic mice being administered with a number of probiotic strains which are distinguishable by their RAPD-fingerprint and samples of feces or biopsies from the GIT of mice could be analyzed to identify the strain with the longest residence time within the host. If administered to individuals with an intact microflora, a pre-selection step with selective media in combination with morphological analyzes would be necessary to select *B. longum*
strains before applying picked colonies to RAPD analyzes. Another approach exploits the potential of probiotic in co-cultures. Co-cultures with bifidobacteria were shown to induce stress tolerance or production of chaperones in *B. longum* and thus the effect of different co-culture combinations or ratios could be analyzed with immobilized cell technique and continuous culture. Biomass of probiotic strains with different probiotic properties could be produced in separate continuous reactors containing bacteria in gel beads and produced under controlled conditions. Biomass produced in immobilized chemostat cultures would be used for controlled inoculation of reactors run in batch mode in co-culture and simultaneously for monoculture in batch mode as reference (Figure 19 A). The system would allow efficient analysis of effect of co-cultures on cell physiology or on gene-/ protein expression. One important benefit of cell immobilization is the ability to control and stability of different strains within a mixed fermentation process (Doleyres *et al.*, 2004b; Lacroix and Yildirim, 2007).

Another experiment would be the exposure of sub-lethal stress additional to cell immobilization in a two-stage fermentation system and cell continuous culture (Figure 19 B). The first reactor is used to efficiently produce active biomass with cells immobilized in gel beads. The released cells from gel beads are pumped in a second reactor where they are exposed to sub-lethal stress treatment similar as used with continuous free cell system recently described and validated by Mozzetti (2009). The advantage of using two-stage continuous system with immobilized cells is the stability and robustness of the system combined with an adjustable (high) dilution rate without running the risk of washing out active biomass. The different stresses cells encounter during growth in gel beads and sub-lethal treatment may lead to a synergistic stress response and result in cross-protection.
Figure 19  Possible fermentation systems to produce probiotic bacteria with enhanced technological and functional characteristics. (A) A number strains are produced using continuous culture with IC in separate systems and effluent used to inoculate mixed batch co-cultures at different proportions for fast screening of strain interaction in the second reactor stages. (B) One strain is continuously produced in a first reactor containing IC and used to inoculate a second reactor, where cells are exposed to sub-lethal stresses (Lacroix and Yildirim, 2007).
6 Appendix

6.1 Supplementary material from Chapter 4

Figure S1   RAPD patterns of 22 *B. longum* strains. Patterns obtained with the primers OPA-02; OPL-07 and OPL-16. M<sub>L</sub>: Low mol. weight DNA marker; M<sub>H</sub>: High mol. weight DNA marker.
6.2 Genome expression analysis of *B. longum* NCC2705 produced during continuous culture with immobilized cells and batch culture

6.2.1 Background

Global gene expression or comparison of protein profiles are valuable tools in the field of comparative analysis between suspended and immobilized bacteria. Numerous studies compared differences of gene expression profiles from cells within biofilms and in planktonic state and reported changes only in small numbers of genes (Whiteley *et al.*, 2001, Schembri *et al.*, 2003). However, only few studies describe the comparison of artificially immobilized microbial cells and cells from free cell culture. Global proteomic analyzes of Gram positive and Gram negative bacteria of sessile (immobilized) cells on various carriers compared to free (planktonic) cells revealed considerable alterations with different expression of detected protein spots ranging from 3 to more than 50% (Junter and Jouenne, 2004).

In our study, transcriptomic analyzes of samples from both ICC cultures and free cells batch cultures were compared among each other to characterize the effect of 2-stage continuous IC cultures with respect to sugar content of the feed, to culture age and to growth in immobilized state. Gene expression patterns were compared with morphological and physiological modifications of cells from IC continuous culture.

6.2.2 Material and methods

Samples were collected from both ICC as described in Chapter 2. Genome wide expression analysis between continuously produced IC cells from R2 was carried only as single experiments
for the following hybridizations: “ICC1 day 8 - Batch (ES)”; “ICC1 R2 day 8 –ICC2 R2 day 8” and “ICC2 R2 day 20 – ICC2 R2 day 8” (Figure S2).

**Figure S2** Hybridization scheme. Each arrow represents one hybridization experiment. Abbreviations: “ES” early stationary and “MS” mid stationary growth phase

Microarray design and RNA extraction DNA based arrays, produced by Agilent Technologies (www.agilent.com), were obtained by *in situ* synthesis of 60 mer oligonucleotides on glass slides (Wolber *et al.*, 2006). For each gene, 3 to 6 different probes were randomly distributed on the array. Total RNA was extracted with the Macaloid method and purified as previously described (Parche *et al.*, 2006).

For each hybridization, cDNA was synthesized starting from 4 µg of total RNA and subsequently labeled using the Array 900MPX Genisphere kit (Genisphere Inc., Hatfield, PE, USA), following the protocol provided by the supplier. Luciferase and kanamycin control
mRNA (Promega, Zurich, Switzerland) at 1 and 10 ng, respectively, were mixed with total RNA before labeling to allow balancing of the two channels during scanning. After the hybridization procedure, array slides were scanned at 10 µm using a Scanarray 4000 (Packard Biochip Technologies, Billerica, MA, USA). Parameters (laser power and photomultiplier tube gain) were set in order to prevent saturation of any spot, except the probes corresponding to rRNA. Data extracted with Imagene 5.6 (Biodiscovery, El Segundo, CA, USA) were treated with homemade scripts in Python language (www.python.org) and a local installation of the ArrayPipe web server (Hokamp et al., 2004). Probes showing a signal smaller than twice the standard deviation of the local background were considered without signal. Probes showing no signal or saturated signals in both channels were discarded from the analysis. Assuming an intensity-dependent variation in dye signal, (limma) loess global normalization was applied on signal ratios. To calculate average gene expression values, data from different probes and hybridization duplicates (with dye swap) were combined as follows. Within each hybridization data set, gene fold changes were calculated from the median of the corresponding probes values. The expression value of a gene was retained if a signal was detected in at least 50 % of its probes. The average gene expression values were then calculated by combining data from two independent hybridizations. Genes were considered to be differentially expressed if their log2-transformed signal ratios were higher than 1.0 or smaller than -1.0. *B. longum* genes organized in operons were identified according to Price et al. (2006) (http://www.microbesonline.org/operons/OperonList.html).
6.2.3 Results and discussion

All hybridization experiments were only carried out once without dye swap confirmation and hence data are of limited significance, but general trends can be elucidated.

6.2.3.1 Comparison of cells from ICC with batch cultures

Cells from the second reactor produced during continuous cultures with immobilized cells were compared with free cells batch cultures from early- and mid stationary growth phase. These comparisons were carried out to find a correlation between gene expression profiles and physiological changes including fatty acid compositions of cell membrane. Gene expression of cells from ICC1 collected at day 8 were compared to cells from early stationary phase and showed a relative expression of 1587 (92 %) ORFs of which 229 (14 %) genes were differently expressed. When compared with cells from mid stationary phase a relative expression of 1347 ORFs (78 %) was reported among which 149 (11 %) were differentially expressed (Table S1). Relative changes in gene expression of cells from continuous IC culture were more pronounced when compared with cells from early stationary batch cells than when compared with cells from mid stationary growth phase (Tables S2 a, b).

Gene expression of cells from ICC1 exhibited less differently expressed ORFs when compared with batch cells from mid- than with cells from early stationary growth phase. This was expected both cultures, from R2 of ICC1 and from batch mid stationary growth phase, face comparable time periods in starvation conditions. ICC cultures indeed showed an increased expression of proteins involved in the ribosomal complex compared to batch free cell cultures and may be a result of sensing environmental changes and lead to modulated synthesis of metabolic enzymes affecting cell physiology (Chen et al., 2003, Wilson and Nierhaus, 2005). Bile tolerant ICC cells
did not show increased transcript levels of neither BL1102 (Na\(^+\) dependent nucleoside transporter), a putative cholate transporter, nor BL0796 (choloylglycine hydrolase), a bile salt hydrolase. Bile tolerance is therefore most likely attributed to changes in membrane composition. These first gene expression profile comparisons showed that cells from ICC did neither show up-regulation of ORFs related to stress response as reported for heat adapted \textit{B. longum} NCC2705 (Rezzonico \textit{et al.}, 2007), nor different expression of (identified) enzymes involved in fatty acid production (Schell \textit{et al.}, 2002). However, it is known that transcript level and proteins content do not necessarily correlate since their relationship depends strongly on time, cellular location, stability of the molecules (Ghigo, 2003) and thus an enzyme-mediated bile resistance in ICC cells cannot be excluded.

\textbf{Table S1} Summary of hybridization experiments. Absolute and relative number of totally expressed, under- and over expressed genes.

<table>
<thead>
<tr>
<th>Hybridization(^a)</th>
<th>Control</th>
<th>Treated</th>
<th>Expr.</th>
<th>Expr. (%)</th>
<th>Over-expr.</th>
<th>under-expr.</th>
<th>Diff. expr.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC1 day 8 - Batch (ES)</td>
<td>Batch (ES)</td>
<td>ICC1 day 8</td>
<td>1587</td>
<td>91.9</td>
<td>102</td>
<td>127</td>
<td>229</td>
</tr>
<tr>
<td>ICC1 day 8 - Batch (MS)</td>
<td>Batch (MS)</td>
<td>ICC1 day 8</td>
<td>1347</td>
<td>78.0</td>
<td>85</td>
<td>64</td>
<td>149</td>
</tr>
<tr>
<td>ICC2 day 8 – ICC1 day 8</td>
<td>ICC1 day 8</td>
<td>ICC2 day 8</td>
<td>1007</td>
<td>58.3</td>
<td>69</td>
<td>101</td>
<td>170</td>
</tr>
<tr>
<td>ICC2 day 20 – ICC2 day 8</td>
<td>ICC2 day 8</td>
<td>ICC2 day 20</td>
<td>1541</td>
<td>89.2</td>
<td>52</td>
<td>47</td>
<td>99</td>
</tr>
</tbody>
</table>

\(^a\) ES: early stationary growth phase; MS: mid stationary growth phase
* Differently expressed, when over-/underexpression \(\geq\) 2 fold.

\subsection*{6.2.3.2 Comparison between ICC1 and ICC2 and within ICC2}

Gene expression profiles of cells from continuous cultures with immobilized cells with and without glucose limitation collected at day 8 were compared and a relative expression value of approximately 58\% (1007 genes) of the ORFs was found with 170 genes (17\%) differently
expressed (Table S1). The most over- and underexpressed genes were both hypothetical protein BL1055a (hypothetical protein) and BL0981 (hypothetical protein with helix turn helix motif), respectively, (Table S3). Among the 10 most repressed genes of cells from ICC2, 4 genes are involved in sugar transport or metabolism. The sugar limitation in ICC1 may induce a considerable increase of sugar transporter proteins compared to cells which are not sugar limited (ICC2) in order to open gates for possible energy resources in the environment.

DNA micro array analysis between cells from ICC2 collected at day 20 containing large cell aggregates were compared to cells collected before aggregation at day 8 and enabled assessment of relative expression value of approximately 89% (1541) of ORFs (Table S1). In total, 99 genes (6%) were differentially expressed. The most over- and underexpressed genes were BL1040 (hypothetical protein) and BL1694 (probable sugar binding protein of ABC transporter for pentoses), respectively (Table S4). Comparison of aggregated cells from ICC2 day 20 showed a down regulation of transporter proteins of pentoses. The membrane proximity of high cell density environment in macroscopic cell aggregates may induce quorum sensing (Sturme et al., 2002) and result in a down-regulation of sugar transport proteins.

Although gene-expression experiments are only preliminary results, the data support some physiological observations and may confer the understanding of molecular mechanisms underlying increased bile tolerance and formation of auto-aggregation. However, further hybridizations are required (including dye-swap experiments) to confirm obtained global gene expression patterns of cells from ICC compared to cells from batch control cultures.
Table S2a  The 10 most over- and underexpressed genes represented by the factor of induction/repression of cells from ICC1 Day 8 compared to Batch ES.

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<td><strong>Over-expressed</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.3</td>
<td>BL1631</td>
<td>D-Glucose-proton symporter</td>
<td>BL1630-1631</td>
</tr>
<tr>
<td>7.5</td>
<td>BL0105</td>
<td>beta-fructofuranosidase (sucrase/invertase); possible inulinase</td>
<td>BL0105-0107</td>
</tr>
<tr>
<td>6.7</td>
<td>BL0106</td>
<td>sucrose transport protein</td>
<td>BL0105-0107</td>
</tr>
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<td>4.9</td>
<td>BL1493</td>
<td>possible WhiB-like transcription factor</td>
<td>BL1493-1494</td>
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<tr>
<td>4.9</td>
<td>BL1494</td>
<td>hypo. protein BL1494</td>
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<td>BL1492</td>
<td>hypo. protein with similarity to the Par protein of B. breve plasmid pcibbl1</td>
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<td>30S ribosomal protein S12</td>
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<td>BL0624</td>
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</tr>
<tr>
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<td>BL1586</td>
<td>50S ribosomal protein L16</td>
<td>BL1577-1604</td>
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<tr>
<td>-9.8</td>
<td>BL0036</td>
<td>probable ABC transporter permease protein for sugars</td>
<td>BL0033-0036</td>
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<td>BL1330-1333</td>
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<td>probable solute-binding protein of ABC transporter system</td>
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<td>-7.0</td>
<td>BL0034</td>
<td>ATP binding protein of ABC transporter</td>
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<td>BL1331</td>
<td>probable sugar permease of ABC transporter system</td>
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<td>BL0813</td>
<td>hypo. protein BL0813</td>
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<td>-6.1</td>
<td>BL1335</td>
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<td>BL1334-1335</td>
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<tr>
<td>-5.7</td>
<td>BL1165</td>
<td>probable solute binding protein of ABC transporter system for sugars</td>
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Table S2b  The 10 most over- and underexpressed genes represented by the factor of induction/repression of cells from ICC1 Day 8 compared to Batch MS.

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<td><strong>Over-expressed</strong></td>
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<td></td>
</tr>
<tr>
<td>6.6</td>
<td>BL1631</td>
<td>D-Glucose–proton symporter</td>
<td>BL1630-1631</td>
</tr>
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<td>BL0555</td>
<td>possible DO serine protease</td>
<td>-</td>
</tr>
<tr>
<td>5.8</td>
<td>BL0105</td>
<td>beta-fructofuranosidase (sucrase/invertase); possible inulinase</td>
<td>BL0105-0107</td>
</tr>
<tr>
<td>5.4</td>
<td>BL0106</td>
<td>sucrose transport protein</td>
<td>BL0105-0107</td>
</tr>
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<td>30S ribosomal protein S8</td>
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<td>BL1595</td>
<td>50S ribosomal protein L6</td>
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<td>BL1598</td>
<td>50S ribosomal protein L30</td>
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<td>BL1596</td>
<td>50S ribosomal protein L18</td>
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<td>BL0358</td>
<td>ATP synthase gamma chain</td>
<td>BL0356-0363</td>
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<td>BL1586</td>
<td>50S ribosomal protein L16</td>
<td>BL1577-1604</td>
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<td><strong>Under-expressed</strong></td>
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</tr>
<tr>
<td>-9.3</td>
<td>BL0036</td>
<td>probable ABC transporter permease protein for sugars</td>
<td>BL0033-0036</td>
</tr>
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<td>ATP binding protein of ABC transporter</td>
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<td>probable ABC transport system permease protein for sugars</td>
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<td>BL0033</td>
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<td>BL1772</td>
<td>sugar kinase in PfkB family</td>
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<td>C4-dicarboxylate transporter</td>
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<td>hypothetical protein BL1773</td>
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Table S3  The 10 most over- and underexpressed genes represented by the factor of induction/repression of cells from ICC2 Day 8 compared to ICC1 Day 8.

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<td>BL0945</td>
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<td>FtsE-like ATP binding protein involved in cell division</td>
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<tr>
<td>4.0</td>
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<td>FtsX-like protein involved in cell division</td>
<td>BL1182-1184</td>
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<td>acetylglutamate kinase</td>
<td>BL1058-1068</td>
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<td>BL1714</td>
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Table S4  The 10 most over- and underexpressed genes represented by the factor of induction/repression of cells from ICC2 Day 20 compared to ICC2 Day 8.

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Over-expressed

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7 References


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